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ERRATA

- Vol. 16, page 210, line 4 from bottom, "f" should read "of"
- Vol. 16, page 294, line 4, "AWAITI" should read "AWATI"
- Vol. 16, page 409, line 7 from bottom, "*Cucurbita*" should read "*Cucumis*"
- Vol. 17, page 105, footnote 2, delete "at Boyce Thompson Institute"
- Vol. 17, page 165, line 4, "aerobic" should read "anaerobic"
- Vol. 17, page 190, TABLE XII, column 1, lines 6 and 12, "*Sclerotinia*" should read "*Monilinia*"
- Vol. 17, page 201, line 8, "Mers." should read "Merr."
- Vol. 17, page 214, line 9, "sulfuramino" should read "sulfur amino"
- Vol. 17, page 222, paragraph 2, line 10, should read "5,7-dichloro-8-hydroxyquinoline" etc.
- Vol. 17, page 228, line 10, "componds" should read "compounds"
- Vol. 17, page 275, TABLE I, column 4, line 6 from bottom, "4-NCl" should read "4 = NCl" and line 3 from bottom, "1,2-O" should read "1,2 = O"
- Vol. 17, page 285, line 21, "fugicides" should read "fungicides"
- Vol. 17, page 322, line 12 from bottom, "76" should read "72"
- Vol. 17, page 335, line 4 from bottom, "*Althea*" should read "*Althaea*"
- Vol. 17, page 336, line 6 from bottom, insert "[New Haven]" after "Connecticut"
- Vol. 17, page 381, line 4 and title of TABLE I, insert "male" before "German"
- Vol. 17, page 413, paragraph 2, line 5, "increased" should read "decreased"

TRANSMISSION OF A RINGSPOT-VIRUS DISEASE OF SYRINGA VULGARIS BY GRAFTING

J. H. BEALE AND HELEN PURDY BEALE

In the summer of 1949, a ringspot disease of the common lilac, *Syringa vulgaris* L., was observed by the authors in the vicinity of Yonkers, New York. They reported at the 7th International Botanical Congress, Stockholm, in 1950 (2) that this was believed to be the first record of its appearance in the United States. Sections of the affected leaf tissue were examined under the microscope and gave no evidence of insect damage, and revealed the presence of no bacteria or fungi. It was noted that the disease closely resembled one described in North Bulgaria by Atanasoff (1) in 1935 in an illustrated article and attributed by him to a virus, although no evidence was presented. Since the diseases of other hosts, caused by viruses, exhibit symptoms similar to those of the ringspot disease of lilac, it was decided to obtain some evidence of its virus nature by attempting to transmit the etiologic agent by grafting. As recorded in a preliminary report (3), the experiments were successful.

SYMPTOMS OF THE DISEASE

Superficially, the lesions resemble the work of a leaf miner. As shown in the leaf at the right in Figure 1, the leaves look as though they were etched with light-green wavy lines, forming ringlike lesions in the veinlet areas, and frequently a single band across the entire leaf blade, or an "oak leaf" pattern, as shown at the base of the leaf, creating the impression that the virus has diffused outward from the larger veins or midrib. The healthy leaf at the left was taken from another variety. The lilac bushes near Yonkers are heavily infected, but in spite of this fact, an occasional twig appears to be altogether free of symptoms, which was found to be a phenomenon characteristic of this disease. In the case of bushes where the virus is not uniformly distributed, it is often necessary to search carefully to detect the ringspot symptoms. With the exception of the lesions on the leaves, just described, the plants appear to be normal. No brittleness or rolling of the leaf margins, and no premature or delayed defoliation occurs, such as is descriptive of lilac mosaic, and the color and appearance of the blossoms are unaffected.

ATTEMPTS TO TRANSMIT THE DISEASE BY GRAFTING

A number of preliminary attempts at rooting and grafting cuttings of the lilac, affected with ringspot, were made by the junior author in 1949 and

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1950, but were not satisfactory. In the fall of 1951, the senior author dug up and potted some suckers of the infected lilacs with their roots attached and used them later for understock or scion. Some cuttings were also rooted, along with those of two varieties of healthy lilac from the Boyce Thompson Arboretum, namely, Mlle. Mélide Laurent and Paul Deschanel, which were obtained from a nursery in France many years ago. In January,

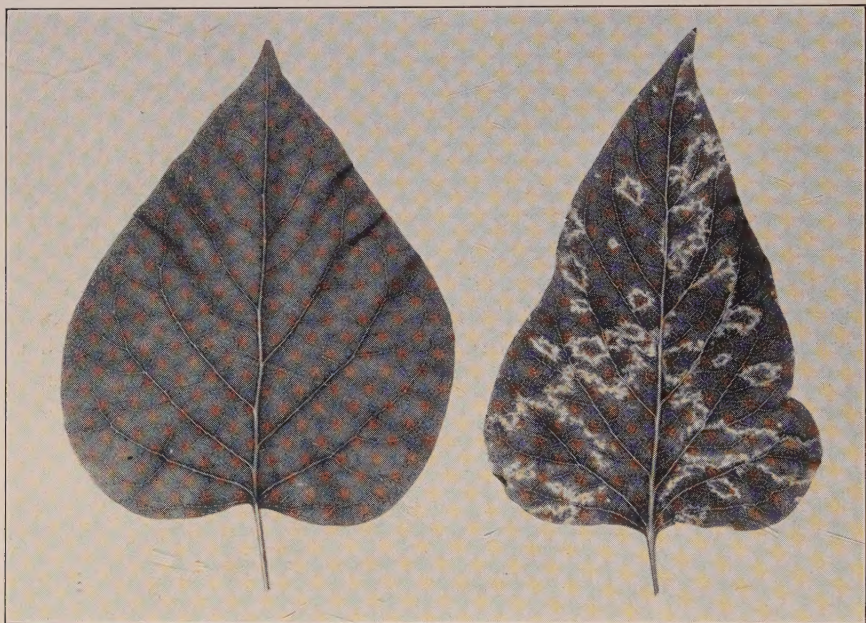


FIGURE 1. Ringspot disease of the common lilac, *Syringa vulgaris* L., showing ringlike lesions and wavy bands, which form an "oak leaf" pattern. The leaf at the left is from a healthy plant of a different variety.

1952, a total of 14 grafts were made. During the previous summer, a number of buds from the affected lilacs were inserted in the two healthy varieties, but no growth took place except in three cases, although in several instances the buds had united and remained green for more than a year, at the time of the last examination.

RESULTS OF THE TRANSMISSION EXPERIMENTS

Where the affected lilac was used as understock and healthy lilac, variety Mlle. Mélide Laurent as scion, successful transmission of the virus was obtained in four out of five grafts, as evidenced by the appearance of typical ringspot lesions on the leaves of the scion. In two cases where the order of the stock and scion was reversed, transmission did not occur, as well as in two instances where the infected lilac was budded on healthy lilac.

In two out of four grafts, where the ringspot lilac was used as understock and the healthy lilac, variety Paul Deschanel, as scion, transmission was obtained from stock to scion. In two out of three cases, where the stock and scion were grafted in the reverse order, transmission took place from the affected scion to the healthy understock, Paul Deschanel. In one instance, the ringspot affected lilac was budded on Paul Deschanel and transmission was effected.

Out of a total of 14 grafts, eight cases of transmission were obtained.



FIGURE 2. (Left to right). The first leaf is from a healthy plant of *Syringa vulgaris* L. var. Paul Deschanel, and the third leaf is from a scion of the same variety grafted on understock of an unidentified variety, affected with the ringspot virus. The wavy lines and rings on the third leaf indicate transmission of virus has occurred from understock to scion. The second leaf is from the affected understock, and the fourth leaf from Mlle. Mélide Laurent shows the yellow blotches of undetermined cause, developing on many of the grafted plants of the three varieties used.

When buds were used, in three cases where growth from the bud occurred, one successful transmission took place. The other two buds grew vigorously, but no ringspot symptoms were present on the leaves produced by the buds at any time, where transmission failed.

In Figure 2, reading from left to right, the second leaf was removed from the understock of common lilac, infected with the ringspot virus. The third leaf was detached from the scion, in this case the variety Paul Deschanel, and the wavy bands and rings typical of the disease have appeared as the result of transmission from the affected understock. The first leaf is from a healthy plant of the same variety, Paul Deschanel. The first and third leaves are darker than the other two in the figure, because they were taken from the growing tip and were thicker and a darker green than

the second and the fourth leaves, removed from the base of the plant where yellowing due to age had begun.

The fourth leaf in Figure 2 shows yellow blotches which appeared, after grafting, on many of the plants. The cause of this symptom has not been determined, but transmission of the virus was recorded as positive only when the typical ringspots or wavy bands were present, although the virus rarely invaded more than a limited area of the healthy plant, judging from the appearance of symptoms.

REPORTED OCCURRENCE OF THE DISEASE

The ringspot-virus disease of the common lilac has been reported from North Bulgaria in 1935 by Atanasoff (1), as previously mentioned, and from Yugoslavia by Nikolić (4), who states that he saw a ringspot disease on lilac in Zemun in 1948 and in Belgrade a year later. Smolák and Novák (5), include a group of the ringspot type among the diseases of lilac reported in Czechoslovakia in 1950. The authors first observed the ringspot disease in New York State in the vicinity of Yonkers in 1949, and in Rochester, New York in 1952, while the junior author found a few cases of the disease in the Arboretum of the University of Wisconsin during the same year. In 1950, the authors also observed a single bush infected with the ringspot virus in Leyburn, England, and another in Voss, Norway.

VARIETIES OF LILAC FOUND TO BE AFFECTED BY THE DISEASE

Infection has been induced in the varieties Mlle. Mélide Laurent and Paul Deschanel, as described herein. Specimens of the latter variety were found to be free of the disease at Highland Park, Rochester. In the summer of 1952, over 100 varieties of lilac were examined for this disease at Highland Park and only five varieties were found to be infected, namely, Ruhm Von Horstenstein, Pyramidalis, Eden, Professor E. Stoeckhardt, and Mrs. E. van Nes. Two large specimens of the variety Ruhm Von Horstenstein were both infected, although they were planted about 150 feet apart. In the Arboretum at Madison, Wisconsin, according to the Director, Mr. G. W. Longenecker, the four diseased varieties all belonged to the same lot of plants, purchased from a nursery in 1935. The affected varieties were Desfontaines, Gloire de Moulins, Hugo Koster, and Président Grévy. Another bush of Président Grévy, obtained at another time from a different source and growing about 20 feet distant from the affected specimen, was free of the disease. Also, Gloire de Moulins, which exhibited pronounced ringspot symptoms at the Arboretum of the University of Wisconsin and was one of this same group of infected plants, was found to be healthy at Highland Park, Rochester.

DISCUSSION

The yellow blotches which occurred quite commonly among the grafted plants, as illustrated in the fourth leaf of Figure 2, might be due to a differ-

ent manifestation of the ringspot virus, or to another virus, or to some physiological disorder. It was not observed on bushes in the field which showed typical ringspot symptoms.

The failure to obtain transmission of the virus as readily by budding as by grafting, may be accounted for by the fact that so much less tissue is employed in the former method that the buds may have been free of virus. In fact, the absence of any ringspot symptoms in the two cases of growth from the buds, where transmission failed, and their presence in the one successful instance, would tend to substantiate this view.

Since the ringspot disease occurs so seldom in old plantings of the common lilac, it seems likely that the disease is not transmitted by pruning and that the insect vector or vectors, if they exist, are probably not present at least in the United States. The vectors may exist in other countries, possibly the Balkans, where the lilac is native, or in Czechoslovakia where, according to published reports, virus diseases of lilacs are quite severe (5).

Since the affected varieties at Madison, Wisconsin, were all purchased from the same source, and that healthy specimens of *Président Grévy*, bought elsewhere, were found close-by, the indication is that they were propagated on diseased understock. The fact that *Gloire de Moulins*, one of the same lot, was affected at Madison but healthy at Rochester, would give added weight to this opinion.

When this disease was found in the United States and in England and Norway, it did not appear to be causing serious damage, but a virus is always a potential danger in that it may mutate and become more virulent, or it may cause more serious damage in another host or in combination with another virus in the original host. Also, there is always the possibility that a vector may be introduced that will spread the virus. The disease was probably introduced from Europe and appears to be perpetuated by infected stock. It could doubtless be controlled by care in the selection of propagating material.

SUMMARY AND CONCLUSIONS

The symptoms of a ringspot-virus disease of the common lilac, *Syringa vulgaris* L., are described and illustrated and their similarity to a virus infection of lilac, occurring in North Bulgaria, is noted. The disease was observed by the authors on three unidentified varieties, the first in the vicinity of Yonkers, New York, and the others on single bushes in Leyburn, England, and in Voss, Norway. It was also found on five named varieties at Rochester, New York, and on four others at Madison, Wisconsin.

In eight out of 14 attempts, the disease was transmitted to two additional varieties of known identity by grafting. Transmission was obtained in one out of three cases by budding.

It is concluded from the similarity of the lesions to other known virus diseases, and from the successful transmission of these symptoms to

healthy plants by grafting, that the etiologic agent is a virus.

The suggestion is made that the disease was introduced from Europe and is probably distributed here solely by infected stock.

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5. SMOLÁK, J., and J. B. NOVÁK. [Virus diseases of lilac.] *Ochrana Rostlin* **23**: 285-304. 1950. (Orig. not seen.) (*Abstr. in Rev. App. Mycol.* **30**: 520. 1950.)

RELATION OF DIFFERENT GASES TO THE SOAKING INJURY OF SEEDS. II.

LELA V. BARTON

In a previous paper from this laboratory (5) it was shown that soaking injury to seeds is enhanced by passing oxygen through the water in which the seeds are soaked. Passing air or nitrogen in like manner reduced the harmful effect but did not permit normal germination. Carbon dioxide treatment, on the other hand, prevented the deleterious effects in many instances. These findings indicated clearly that any disorganization of the metabolism of the seed resulting from soaking is due neither to a deficiency in the oxygen supply nor to an accumulation of carbon dioxide, two explanations of soaking injury given by previous workers. Preliminary tests to determine the cause of the gas effects eliminated acidity, bacterial action, and leaching of essential materials as being solely responsible, but there was a striking reduction in the rate of moisture absorption under treatment with carbon dioxide as compared with the other gases tried (non-aerated, air, nitrogen, or oxygen). It was stated that there may be a direct relationship between the amount of moisture absorbed and injury from soaking (5).

The data to be given in the present paper represent an extension of studies emphasizing the harmful effect of oxygen and the protective effect of carbon dioxide in soaking experiments where salt solutions as well as water were used. Additional evidence of the importance of moisture absorption in relation to these effects is shown by the protective action of inert polyvinylpyrrolidone which reduces the moisture absorption rate when harmful oxygen is supplied during soaking, but is without effect in the presence of carbon dioxide. Extended tests have shown that carbon dioxide not only protects against injury to seeds by soaking in water, but also against the deleterious action of certain salt solutions. This is directly related to the possibility of incorporating inorganic fertilizers, both major and minor elements, with the seeds before planting, a problem which has received considerable attention by other people but which has not proved consistently successful on a commercial scale because of the toxic effects of the salts on the seeds themselves. Carbon dioxide can also prevent injury to seeds by solutions of such toxic substances as sodium selenate and 2,4-dichlorophenoxyacetic acid. A possible new method for the evaluation of germination energy of corn samples consists of soaking the grains in water supplied with oxygen for 24 hours before planting.

It should be kept in mind that the gas effects to be described in this

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paper are all to be found in the pretreatment of seeds before planting. No control of gaseous exchange was made during the actual germination process or during the growth of the seedlings.

MATERIALS AND METHODS

The seeds used in the principal experiments were: oat, *Avena sativa* L. variety Clydesdale; tomato, *Lycopersicon esculentum* Mill. variety Bonny Best; rice, *Oryza sativa* L., obtained through the courtesy of Dr. L. H. Flint of Louisiana State University, Baton Rouge, La.; bean, *Phaseolus vulgaris* L. variety French Horticultural; wheat, *Triticum aestivum* L. variety Marquis; and corn, *Zea mays* L. Pioneer Hi-Bred (335 large flat).

Polyvinylpyrrolidone used in the experiments was obtained through the courtesy of General Aniline and Film Corporation.

The method generally used was to soak counted lots of seeds in measured amounts of water or salt solutions at laboratory temperature (21° to 26° C.) and treat with different gases during the soaking period. The gases used were air, oxygen, nitrogen, and carbon dioxide. Air from the laboratory was pulled through the soaking solutions by means of a vacuum. Oxygen, nitrogen, and carbon dioxide were supplied from cylinders of these gases. All gases were allowed to bubble slowly through the solutions containing the seeds for the test period, then the solutions were drained from the seeds which were used for germination and growth tests and chemical analyses. Two types of controls were used, dry seeds and seeds which had been soaked in non-aerated water.

Measurements of the amount of phosphorus and potassium taken up by seeds were made according to the method of Wolf and Ichisaka (21), using a Klett-Summerson photoelectric colorimeter.

The amount of water or solution absorbed by the seeds was measured by determining the increase in weight of counted weighed lots of seeds after soaking for different lengths of time. A uniform procedure was followed for removing all surface moisture from the seeds before the sample was placed in a weighing bottle and weighed to 0.1 mg.

The soil used in greenhouse tests was deficient, and for the most part was made up of $\frac{1}{2}$ sod soil, $\frac{1}{4}$ granulated peat moss, and $\frac{1}{4}$ sand, in 6-inch glazed crocks. River sand was used in one test, and sod soil alone in another.

RESULTS AND DISCUSSION

INJURY EFFECTS AS RELATED TO MOISTURE ABSORPTION

Effect of polyvinylpyrrolidone (PVP). PVP is a synthetic polymer which may prove useful as a substitute for blood and plasma when these are unavailable for treatment of shock. It has also been used as a retardant vehicle for drug effects, and has been reported (14) to retard or inhibit ab-

sorption of water by branches of eucalyptus and lilac. Also, it has been found that a 5 per cent solution retards the germination of peas and 10 to 20 per cent prevents germination without injuring the seeds which germinate normally upon being removed to water (14).

If differences in injurious effects of gases are to be accounted for in part by excessive moisture absorption, and PVP retards the absorption of water, less injury should result in any given case in solutions of PVP than in water.

Preliminary experiments to test this hypothesis were conducted with

TABLE I
ABSORPTION OF AERATED SOLUTIONS OF POLYVINYLPIRROLIDONE (PVP) BY
BEAN SEEDS AND ITS EFFECT ON GERMINATION

Soaking treatment		Solution absorbed and germination after hours of soaking							
		2		4		8		16	
% PVP	Gas	Solution absorbed, %	Germ., %	Solution absorbed, %	Germ., %	Solution absorbed, %	Germ., %	Solution absorbed, %	Germ., %
1	None	56	86	80	52	92	46	98	18
	O ₂	47	96	86	42	103	20	112	0
	CO ₂	22	100	33	90	49	98	80	98
5	None	40	90	59	98	77	90	89	32
	O ₂	43	90	63	94	87	82	105	6
	CO ₂	23	98	31	100	49	96	80	96
10	None	39	84	55	90	73	84	85	64
	O ₂	37	76	59	86	79	90	86	66
	CO ₂	23	98	32	100	43	98	72	96

beans which were soaked 24 hours in 5 and 10 per cent PVP solutions through which oxygen, nitrogen, or carbon dioxide were passed. The soaked seeds, which had been washed and germinated on moist paper towels at a daily alternating temperature of 20° to 30° C., showed a protective action of the PVP against the germination injury induced by oxygen and nitrogen treatments. In tap water with oxygen, for example, germination was 0 per cent; in 5 per cent PVP, 4 per cent; and in 10 per cent PVP, 76 per cent. No injury from either water or PVP solutions resulted when carbon dioxide was supplied during soaking, and nitrogen-treated seeds held an intermediate position with respect to germination ability.

A more extensive experiment to test these effects was performed, the results of which are shown in Table I and in Figure 1. One, 5, and 10 per cent solutions of PVP were used for soaking beans and determining the amount of water or solution taken up after 2, 4, 8, and 16 hours. Weights were taken of the dry seed lots and of the seeds at the end of each of these

soaking periods, after which samples were planted in soil in the greenhouse for germination tests. Duplicates of 25 seeds each were used.

The percentages of moisture absorbed and the germination of the treated seeds were determined. The absorption of 1 per cent PVP by bean seeds is of the same order of magnitude as the absorption of water measured in previous tests (5). Thus if the amount of moisture absorbed is related to injury, the germination of the seeds after soaking in 1 per cent PVP should have been about the same as after soaking in water. Such was, in fact, the

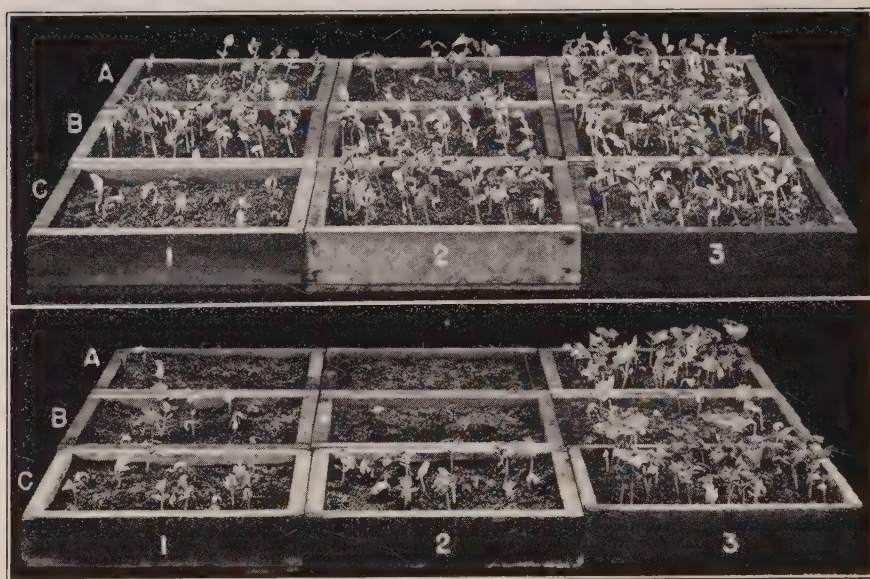


FIGURE 1. The effect of PVP solutions, at concentrations of (A) 1 per cent, (B) 5 per cent, and (C) 10 per cent, on gas injury to bean seeds during soaking periods of 8 hours (above) or 16 hours (below). Gas supplied during soaking: (1) none, (2) oxygen, (3) carbon dioxide.

case. The presence of oxygen caused increasing moisture absorption paralleling increased injury with soaking time as measured by germination ability (Table I). Soaking for as long as 16 hours in the presence of oxygen prevented germination altogether. In non-aerated solutions the seeds were injured less, while in carbonated solutions the injury was prevented. Five per cent PVP reduced the absorption and the injury inflicted by non-aerated or oxygenated solutions. With 10 per cent PVP solutions this reduction was even more marked. It will be noted that absorption from solutions supplied with carbon dioxide was practically the same regardless of the concentration of PVP, and the germination percentages also remained unchanged. Photographs of some of these effects are to be seen in

Figure 1, which shows plants produced from seeds soaked 8 and 16 hours.

These findings tend to confirm the belief that excessive absorption of moisture in the presence of oxygen is a factor in bean seed deterioration, and conversely that the prevention of this excess moisture absorption by the presence of carbon dioxide may account, at least in part, for the protective action against such injury.

Effect of a vacuum and surface-active agents. Forcing more rapid absorption by using a vacuum to pull some of the air out of the seeds immediately after placing them in water or after soaking for 1, 2, or 6 hours made no difference in the relative effects of oxygen and carbon dioxide as far as germination capacity was concerned. Also the use of surface-active agents did not increase the injury to beans when carbon dioxide was bubbled through the soaking water. On the other hand, concentrations of 20 and 320 p.p.m. of Tween 20 (polyoxyethylene sorbitan monolaurate), Vatsol OT [di-(2-ethylhexyl) sodium sulfosuccinate], and Isonol DL1 (dilauryldimethylammonium bromide as a 75 per cent solution in isopropanol), which injured the seeds in non-aerated water or in water supplied with air, oxygen, or nitrogen, had no effect on the germination of seeds presoaked in water supplied with carbon dioxide. In this case, as in many others to be discussed in other sections of this paper, the presence of carbon dioxide protected seeds from toxic substances. This introduces a new favorable effect of the carbon dioxide, but data will show that this action, also, may be partly a matter of the control of absorption of toxic solutions by seeds.

Absorption of water by rice seeds. Since it had been found that oxygen supplied during soaking was harmful to several different kinds of seeds (5), it was of interest to determine whether rice seeds which germinate normally under water would be similarly affected.

Rice seeds were not injured by soaking 4, 8, 16, 24, and 48 hours in water supplied with air, oxygen, nitrogen, or carbon dioxide. Germination began in the soaking water treated with air, oxygen, or nitrogen. Carbon dioxide-treated seeds were somewhat delayed in germination even after removal from the soaking water. Four hours in carbon dioxide caused a shorter delay in germination than longer periods. The question arose as to whether the delay after carbon dioxide treatment was due to retarded water absorption.

The results of an experiment to determine this point are shown in Table II. Weighed lots of 200 seeds each were used. At the end of the appointed soaking period, the seeds were weighed again to determine moisture absorption and then germinated. The difference in moisture absorption with carbon dioxide or with other gases supplied is not so great as in the case of bean seeds. After 24 hours of soaking there were some germinations in all intact seeds except those with carbon dioxide supplied in which case none of the seeds were germinating. With coats removed all seeds were

germinating at this time in the presence of oxygen, but none in the presence of carbon dioxide. After 48 hours, intact seeds were practically all germinated in all gases except carbon dioxide. Removal of the outer coats speeded up the germination in the presence of oxygen over that of intact seeds, but still permitted no germination in the presence of carbon dioxide. Carbon dioxide may delay germination partly by restricting water absorption; however, 29 per cent moisture, demonstrated in other cases to be sufficient for germination, failed to permit germination in rice seeds with coats removed until the seeds ceased to be exposed to carbon dioxide. This

TABLE II
ABSORPTION OF WATER BY 200 RICE GRAINS IN AERATED SOLUTIONS

Seed coats	Gas supplied during soaking	Per cent absorption after hours of soaking			
		6	12	24	48
Intact	None	19.0	23.0	28.4*	33.0*
	Air	18.8	23.6	28.8*	40.4*
	O ₂	18.7	25.4	30.9*	47.1*
	N ₂	18.4	23.0	27.7*	32.0*
	CO ₂	17.3	22.0	26.3	27.6
Removed	O ₂	26.6	29.4	37.2*	58.6*
	CO ₂	25.9	28.3	28.9	29.0

* Grains germinating.

is further evidence, as has been pointed out before (5), that important as the absorption of water appears to be, some of the gas effects are independent of the effects of moisture absorption.

Additional work needs to be done with seeds of other water plants to determine their response to soaking treatments. Rice is the only seed studied so far which failed to show injury from oxygen supplied during the soaking process.

EVALUATION OF CORN SAMPLES

The real measure of the commercial value of a given lot of corn for seed purposes is its performance in the field. Routine tests of seeds which are carried out under controlled conditions in the laboratory or greenhouse do not always give a correct indication of their behavior in the field, especially under adverse conditions. A cold test has been devised to determine the suitability of corn seeds for field planting (20). This test consists essentially in planting the seeds in moist soil infested with seed decay organisms and leaving them for periods up to two weeks at a temperature of approximately 10° C., after which they are transferred to a greenhouse and seedling counts are taken. The survival after such treatment is good for seed lots of high germination vigor, but poor for lots of inferior quality.

The relation of gas supply during a period of presoaking to the cold test for corn has been determined. Fifty seeds each were soaked for 6 and 24 hours in 100 ml. tap water at 10° C. or in the laboratory, non-aerated and aerated with air, oxygen, or carbon dioxide. A similar treatment was carried out in the presence of soil instead of water. Fifty seeds each were mixed with approximately 225 ml. of soil and this mixture placed in aera-

TABLE III
EFFECT OF PRESOAKING CORN SEEDS ON THEIR RESISTANCE TO THE COLD TEST AS INDICATED BY SEEDLING PRODUCTION IN SOIL IN THE GREENHOUSE

Presoaking treatment			Per cent seedling production after days at 10° C. following presoaking					
			Presoaking in tap H ₂ O			Presoaking in soil		
			0	6	12	0	6	12
10° C.	6	None	96	86	58	96	86	28
		Air	94	66	60	100	90	58
		O ₂	98	74	64	100	98	70
		CO ₂	98	72	50	100	98	76
	24	None	100	82	62	94	72	42
		Air	96	70	54	94	84	56
		O ₂	84	56	50	100	82	54
		CO ₂	94	52	36	98	84	56
Lab.	6	None	98	58	46	94	78	48
		Air	98	66	30	96	80	38
		O ₂	88	54	6	96	76	40
		CO ₂	98	68	50	98	74	22
	24	None	100	82	66	96	72	18
		Air	98	80	40	92	74	26
		O ₂	34	10	1	90	78	54
		CO ₂	96	58	48	95	74	20

tion flasks and enough water added to saturate the soil. Gas was passed over and through the soil by means of a glass tube extending about an inch below the surface of the soil into a hole made in the soil in the center of the flask. For exposure at 10° C. all materials including the gas cylinders were placed at this temperature 24 hours before the experiment was started. After each treatment 50 seeds were planted in each of three small flats, one of which was placed in the greenhouse and two at 10° C. The latter were transferred to the greenhouse after 6 and 12 days' exposure to the low temperature. Flats of soil and water for wetting soil were placed at 10° C. or in the greenhouse 24 hours before plantings were made. Seeds in soil treatment in the laboratory for 24 hours had started to germinate in non-aerated and oxygenated flasks. In aerated and carbon dioxide-treated flasks no roots were apparent. The germinated seedlings were planted along with ungerminated seeds.

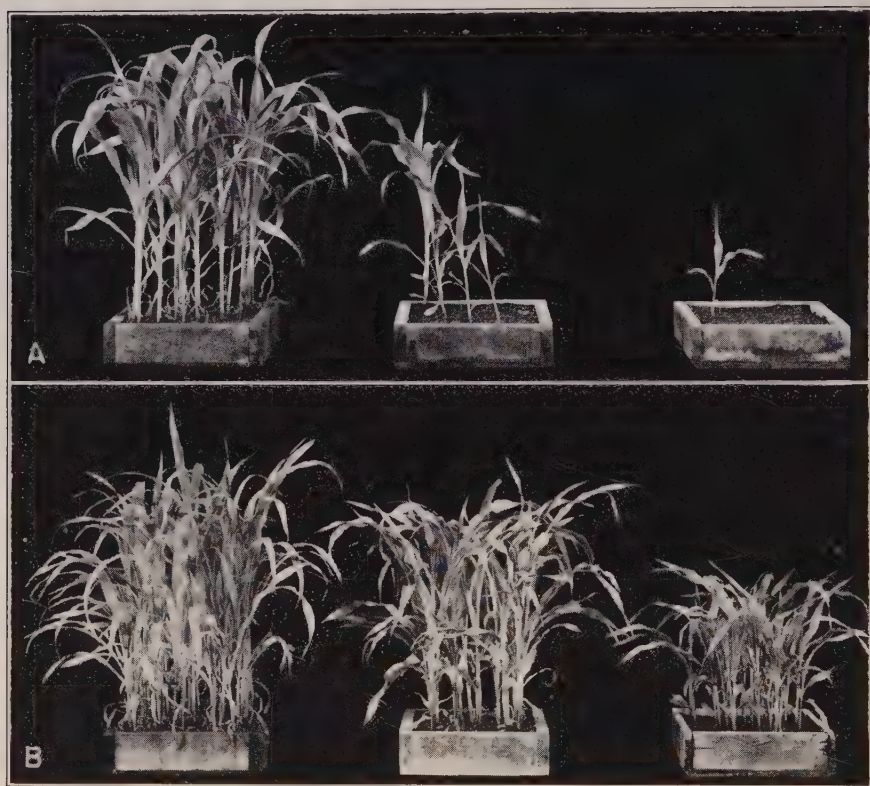


FIGURE 2. Corn seeds soaked 24 hours in tap water aerated with (A) oxygen, and (B) carbon dioxide. Left to right: planted in the greenhouse immediately and after 6 and 12 days at 10° C. Planted August 17; photographed September 13.

The results of these tests are shown in Table III and in Figure 2. When the seeds were planted in the greenhouse immediately after soaking, there were no differences between soaking in non-aerated water, or with air or carbon dioxide supplied, but aeration with oxygen caused greater damage after soaking in the laboratory than at 10° C. Seeds soaked in the laboratory with oxygen also showed least resistance to subsequent planting in cold soil. This is strikingly demonstrated for the 24-hour soaking period by the results shown in Figure 2, where oxygen and carbon dioxide effects are compared. These photographs were taken 27 days after planting. Germination in soil of the vigorous seeds used in these tests could be reduced from approximately 100 to 34 per cent by presoaking in the presence of oxygen for 24 hours (Table III).

It will be noted that oxygen was not damaging to seeds presoaked in saturated soil, but, indeed, may have had the opposite effect when laboratory temperature was used.

Presoaking of corn seeds for 24 hours in the presence of oxygen and then planting in soil may serve some of the purposes for which cold testing is now used; i.e. evaluation of insecticides and fungicides, corn processing methods, and inherited differences in various strains (11, 12, 20). In spite of a considerable amount of work on the subject, the cold testing procedure has not been standardized so that different laboratories get the same results. The oxygen-soaking test is simpler and therefore should be more easily standardized.

EFFECT OF SOAKING IN SALT SOLUTIONS

Color effects. Soaking in water supplied with oxygen resulted in color changes, especially in the case of bean seeds, which were associated with the deterioration of the seeds. Soaking in solutions of K_3PO_4 at concentrations of 2 M or below in the presence of carbon dioxide caused the development of a deep, bright orange color in yellow corn seeds. The color developed within a few minutes and reached its most intense point within 30 minutes. This color change did not occur with a concentration of 4 M of the same salt, nor for other salt solutions or gas treatments. Oat seeds, on the other hand, were light colored in CO_2 - K_3PO_4 treatment and very much darker yellow in the same solutions with other gases. Bean seeds soaked in sodium molybdate took on a deep, bright orange color in the non-aerated and carbonated solutions. This was in contrast to the dark brown color characteristic of oxygen injury to bean seeds soaked in water. The significance of these color changes is not known at the present time. In the case of 4 M K_3PO_4 supplied with carbon dioxide, salt crystals formed on the sides of the aeration flasks above the solutions. This was not noted for any other gas or solution.

Major elements. Injury to the embryo usually prevents the incorporation of needed fertilizers with seeds by soaking before planting. Small amounts of absorbed chemicals are without effect on the growth of the seedlings and larger amounts retard or inhibit germination. Since carbon dioxide has been shown to prevent certain deleterious effects of soaking in water, an experiment was planned to see whether the same protective effect would be evident when seeds were soaked in solutions of fertilizer salts.

In the first test tried, corn, oat, bean, and wheat seeds were soaked in tap water or in solutions containing 0.5 M or 1.0 M KNO_3 , or 1.0 M K_3PO_4 at laboratory temperature. Some of the solutions were non-aerated while others had air, oxygen, nitrogen, or carbon dioxide bubbled through continuously during the soaking period. Seeds were soaked 4 or 24 hours, drained and sown in soil. Duplicate lots of 25 seeds each for bean and corn and 50 seeds each for oat and wheat were used. Dry seeds were used as controls.

Results are shown in Table IV. Data on wheat seeds have been omitted

since their response was essentially the same as that of oat. A striking thing to be noted is the harmful effect of the phosphate. This is not surprising in view of the strong alkaline reaction of this solution. Oat seeds were injured more by the 24-hour than by the 4-hour soaking period. Another effect is to be seen in the protection afforded all the seeds by passing carbon dioxide through the solutions during the soaking period. This same effect is shown by bean seeds soaked in solutions of potassium

TABLE IV
SEEDLINGS PRODUCED IN THE GREENHOUSE FOLLOWING SOAKING OF
SEEDS IN THE LABORATORY

Seed	Gas supplied during soaking	Per cent seedling production following soaking for							
		4 Hr.				24 Hr.			
		Tap H ₂ O	KNO ₃		K ₃ PO ₄	Tap H ₂ O	KNO ₃		K ₃ PO ₄
			0.5 M	1.0 M			0.5 M	1.0 M	
Corn	None	98	98	96	0	100	96	94	0
	Air	98	98	98	0	98	96	84	0
	O ₂	100	96	96	0	78	82	74	0
	N ₂	100	98	100	0	100	96	92	0
	CO ₂	100	98	100	24	100	88	92	20
Oat	None	98	89	87	5	97	85	78	0
	Air	93	92	87	7	98	89	79	0
	O ₂	93	96	93	0	64	79	55	0
	N ₂	87	92	90	1	93	94	76	0
	CO ₂	89	89	81	57	88	84	67	51
Bean	None	78	8	2	0	76	4	12	0
	Air	88	8	2	0	64	2	0	0
	O ₂	36	4	0	0	0	0	0	0
	N ₂	70	8	0	0	68	0	0	0
	CO ₂	100	84	84	0	98	56	52	0

* 0.5 M K₃PO₄ for bean.

nitrate. The harmful effect of oxygen is also shown for corn and bean in this experiment. Bean seeds showed typical sensitivity to both gases and solutions. For salt solutions, as well as for water, it is here demonstrated that carbon dioxide does something besides exclude the harmful oxygen, since nitrogen does not have the same protective action as carbon dioxide. Dry seeds planted as controls yielded 99, 95, and 99 per cent germination respectively for corn, oat, and bean, so the seeds were of high quality. These same seed lots were used for all the experiments to be reported below.

Another series of tests with K₃PO₄ and Na₃PO₄·12H₂O at 0.125, 0.25, and 0.5 M, and a nutrient solution made up of 0.02, 0.04, or 0.08 M of each of KH₂PO₄, Ca(NO₃)₂·4H₂O, and MgSO₄·7H₂O was performed in which 50 bean and corn, and 100 oat seeds were soaked for 1, 2, or 4 hours in non-

TABLE V
SEEDLINGS PRODUCED IN THE GREENHOUSE FOLLOWING SOAKING OF
SEEDS IN THE LABORATORY

Seed	Soaking		Per cent seedling production following soaking in									
	Hr.	Gas	Tap H ₂ O	Na ₃ PO ₄ · 12H ₂ O (M)			K ₃ PO ₄ (M)			Nutrient (KH ₂ PO ₄ ; Ca(NO ₃) ₂ · 4H ₂ O; MgSO ₄ · 7H ₂ O)		
				0.125	0.25	0.50	0.125	0.25	0.50	0.02	0.04	0.08
Corn	1	None	95	85	55	15	80	70	30	100	80	95
		O ₂	95	75	60	5	60	60	20	95	95	90
		CO ₂	100	75	70	60	85	65	60	100	95	85
	2	None	85	20	0	0	50	45	0	75	75	100
		O ₂	90	40	0	0	70	15	0	85	80	75
		CO ₂	75	45	45	40	75	30	25	85	90	85
	4	None	95	40	15	0	65	40	0	85	90	90
		O ₂	100	15	0	0	25	10	0	100	95	100
		CO ₂	95	85	25	45	80	55	25	85	90	85
Bean	1	None	85	15	0	5	20	5	10	85	90	75
		O ₂	70	15	10	5	0	10	10	70	65	95
		CO ₂	85	60	70	70	55	75	60	45	75	75
	2	None	60	5	0	5	0	10	5	75	80	60
		O ₂	90	10	0	5	5	0	10	75	100	40
		CO ₂	100	30	25	10	50	55	25	90	95	90
	4	None	55	0	0	0	0	0	0	40	65	90
		O ₂	35	0	0	5	0	0	0	35	25	20
		CO ₂	95	25	15	15	75	15	10	40	90	90
Oat	1	None	98	84	70	70	92	58	64	94	98	94
		O ₂	96	92	74	70	88	62	80	100	94	100
		CO ₂	100	96	90	74	96	78	64	100	92	96
	2	None	98	66	66	56	88	80	64	94	100	98
		O ₂	94	84	72	56	78	76	66	96	100	98
		CO ₂	100	86	70	70	94	76	68	100	100	100
	4	None	96	78	62	42	76	70	64	100	96	98
		O ₂	94	80	78	44	86	64	50	94	98	98
		CO ₂	96	90	62	60	90	90	72	100	100	100

aerated solutions or those through which oxygen or carbon dioxide was bubbled. Germination percentages of seeds so treated are shown in Table V. Except for beans in the presence of oxygen, the nutrient solutions were without effect on subsequent germination. Tribasic sodium phosphate appeared somewhat more harmful than the potassium salt.

A greenhouse experiment was set up to determine the possible fertilizing effect on growth of plants produced from oat and corn seeds soaked in various chemicals. Fifty-five seeds each were soaked in 100 ml. of solution for four hours at 27° C. in tap water, 0.25 and 0.5 M K₃PO₄, 0.5 M KH₂PO₄,

and 1.0 M KNO_3 non-aerated and supplied with oxygen, nitrogen, and carbon dioxide. Solutions were drained off at the end of the soaking period, and the 55 seeds from each treatment were then placed in a 9-cm. Petri dish on three layers of filter paper which absorbed the excess solution. Each seed touched the filter paper on one side. Plantings in the greenhouse were made from these dishes in replicates of 10 seeds each in an arrange-

TABLE VI

GERMINATION, PHOSPHORUS AND POTASSIUM ABSORBED, AND WEIGHTS OF PLANTS PRODUCED FOLLOWING SOAKING OF CORN AND OAT SEEDS FOR FOUR HOURS

Soaking		Corn					Oat				
Soln.	Gas	Germ., %	P.p.m.		Dry wt.,* g.	Wet wt.,* g.	Germ., %	P.p.m.		Dry wt.,* g.	Wet wt.,* g.
			P	K				P	K		
Tap H ₂ O	None	98	1	161	13.8	103.5	98	1	43	4.8	41.3
	O ₂	98	0	133	16.8	116.0	96	1	41	4.6	38.2
	N ₂	100	1	144	14.6	113.2	90	1	54	4.9	42.2
	CO ₂	98	1	134	15.1	114.1	90	1	49	4.8	42.5
0.25 M K ₃ PO ₄	None	88	6	334	15.2	111.9	60	9	270	6.2	52.0
	O ₂	84	5	357	15.8	117.7	74	12	260	5.6	48.8
	N ₂	88	5	199	17.5	126.4	68	9	312	5.1	43.0
	CO ₂	98	4	297	16.2	117.8	80	7	250	5.4	45.2
0.5 M K ₃ PO ₄	None	16	10	366	13.8 _a	120.9 _a	70	28	246	6.1	51.1
	O ₂	14	13	423	27.7 _b	225.2 _b	64	17	309	6.5	53.3
	N ₂	14	13	450	49.3 _c	338.0 _c	50	15	286	5.9	51.4
	CO ₂	78	8	386	15.8	116.5	62	13	340	5.5	47.8
0.5 M KH ₂ PO ₄	None	96	7	229	16.9	121.0	92	11	156	5.7	48.5
	O ₂	100	8	189	16.8	118.7	88	19	186	4.9	42.0
	N ₂	94	7	230	17.5	131.4	94	11	306	5.6	45.6
	CO ₂	100	7	121	15.4	110.8	92	15	343	4.7	42.6
1.0 M KNO ₃	None	96	1	340	15.0	114.5	80	1	282	5.1	44.4
	O ₂	96	0	280	14.7	113.5	88	0	334	4.5	39.9
	N ₂	98	1	429	15.2	116.5	88	1	352	5.2	44.9
	CO ₂	100	1	272	14.7	109.7	82	1	334	5.5	47.3
Dry control		98	1	193	13.6	112.1	98	1	73	5.4	45.8

* Dry and wet weights are sums of averages for a total of 25 plants (5 pots containing 5 plants each) except: *a.* 8 plants in 3 pots; *b.* 7 plants in 4 pots; *c.* 7 plants in 4 pots.

ment taken from Youden (22) for 21 treatments with 5 replications to permit statistical analysis of growth data to be discussed in another section. Other experiments in which solutions and soaking times were varied somewhat gave the same general results. Solutions used included 0.85 and 1.7 M KH_2PO_4 , 1, 2, and 4 M NaH_2PO_4 , 1 M Na_2HPO_4 , and 0.5 M Na_3PO_4 . Germination records were taken, some of which are shown in Table VI, along with other data from this planting.

It will be seen that the germination of all the lots of corn was good (84

to 100 per cent) except after soaking in 0.5 M K_3PO_4 where non-aerated, oxygen, nitrogen, and carbon dioxide treatment resulted in 16, 14, 14, and 78 per cent germination respectively. Here again is demonstrated the protective action of carbon dioxide. Oat seeds were not so sensitive to injury by the salt solutions and hence the carbon dioxide effects were less striking. The amount of chemical taken up by these seeds and the further growth of seedlings in these plantings as well as germination effects induced by soaking in solutions of trace elements will be described in separate sections. To see the comparative effects of soaking corn seeds in different quantities of 1, 2, and 4 M K_2HPO_4 , and the same concentrations of K_3PO_4 , 55 seeds placed in 25 and 50 ml. of solution were soaked for 15 and 30 minutes. At the end of the soaking periods the solutions were drained from the seeds which were then planted immediately in duplicate 6-inch pots in soil. In 4 M solutions the seeds apparently did not absorb any of the liquids, but a considerable amount of the solutions adhered to the seed at the time of planting.

There was no impairment of germination under any condition with 25 ml. of soaking solution. With 50 ml., 30 minutes' soaking in 2 M K_3PO_4 reduced germination to 58 and 78 per cent respectively when oxygen or nitrogen was supplied. There may have been some reduction even with 15 minutes' exposure to these conditions after which 86 and 84 per cent germination was obtained as compared to the usual near 100 per cent. Gases seemed to be without effect in these short soaking periods. That the quantity of solution used is important has been shown by Roberts (17) and others.

Seeds of corn can be dried for at least 24 hours after soaking in solutions of 0.5 M K_3PO_4 without any impairment of germination. Even when the original germination has been reduced to 26 per cent as a result of the soaking treatment, drying did not seem to injure the seeds further (Table VII). More extensive tests in which corn seeds were soaked in tap water or 0.25 M K_3PO_4 for 0.5, 1, 2, 4, 8, and 16 hours non-aerated and with CO_2 supplied also showed toleration to drying for 2, 4, and 24 hours without additional injury to the seeds.

The amounts of phosphorus and potassium absorbed by or held on the surface of the seeds soaked under various conditions were determined to see whether there is a relationship between these values and the subsequent growth of the plants. From the data in Table VI it will be seen that corn seeds contained up to 13 p.p.m. of phosphorus after soaking as compared with 1 p.p.m. in the dry seeds or in those soaked in water. Large amounts of potassium were recovered from treated seeds, the actual amount varying with the solution and the gases used. It will be noted for 0.5 M K_3PO_4 that there was less absorption of phosphorus in the presence of carbon dioxide. An accumulation of 10 or 13 p.p.m. of phosphorus by the seed during

soaking in this solution is associated with greatly reduced germination (14 to 16 per cent). The presence of as much as 8 p.p.m. of phosphorus decreased the germination from approximately 100 to 78 per cent.

Although salts held on the outside of the seed as well as on the inside would be available for use by the young seedlings, it seemed desirable to determine how much was actually absorbed by the seed. If the chemicals are held on the outside only, the chance of their being lost in the process of planting is very great and could account for the varied reports in the

TABLE VII
GERMINATION PERCENTAGES AND PHOSPHORUS ABSORBED FOLLOWING SOAKING OF
CORN SEEDS IN 0.5 M K_3PO_4

Soaking		Germination, %		P (p.p.m.)	
Hr.	Gas	Wet seeds	Dried seeds*	Without washing	After washing
0.5	None CO ₂	98 100	96 96	7.6 6.0	4.8 3.0
1	None CO ₂	92 96	84 90	8.2 7.4	5.4 4.0
2	None CO ₂	76 88	92 98	11.0 5.8	7.3 3.7
3	None CO ₂	66 92	46 84	20.6 5.9	7.5 3.2
4	None CO ₂	26 92	40 92	12.0 7.0	8.6 3.6

* Dried 24 hours before planting.

literature about the efficacy of the soaking treatment. In order to obtain more specific information on this point, corn seeds were soaked in 0.5 M K_3PO_4 for 0.5, 1, 2, 3, and 4 hours in solutions non-aerated and with CO₂ supplied. At the end of each of these periods the solution was drained from duplicate lots of 10 grams (about 25 seeds), one of which was spread to dry in the manner described above without washing, while the other was washed with 250 ml. of distilled water poured slowly through a perforated dish containing the seeds. Corresponding germination tests were made with duplicates of 25 seeds each. The results are shown in Table VII. It will be seen that much of the phosphorus is lost by washing, but the amount remaining after washing even after CO₂-treatment is at least three times that present in the untreated seed (Table VI). These data show a small absorption under the influence of CO₂ with reduced soaking time of only 30 minutes. As the soaking period was lengthened, the difference became more marked. It is seen that germination was reduced as the phosphorus

content of the seed approached 8 p.p.m. A further decrease in germination of seeds containing 8.6 p.p.m. of phosphorus was evident.

Trace elements. Limited tests have been conducted to determine the effect of soaking seeds in solutions of trace elements. Bean, corn, oat, rice, and tomato seeds were soaked in 0.1 per cent solutions of manganese sulfate, sodium molybdate, copper sulfate, and zinc sulfate in distilled water and in nutrient solution. Distilled and tap water were used as controls. After treatment with no gas, oxygen, and carbon dioxide supplied during soaking, 25 seeds each were planted, without washing, in river sand in gallon crocks. Some precipitate was noted in the 0.1 per cent copper sulfate in nutrient solution, but none appeared in any of the others.

The germination of corn, rice, and tomato seeds was not seriously affected by any trace element solution. Oat seeds were killed by the copper sulfate in distilled water and their germination was reduced about 50 per cent by copper sulfate in nutrient solution when oxygen was supplied during soaking. Slightly less injury resulted from the same solutions non-aerated or with carbon dioxide supplied. Bean seeds were greatly injured by all solutions except when carbon dioxide was supplied. Carbon dioxide did not remove all the soaking injury for copper sulfate solutions, but reduced it enough to permit 12 per cent germination of beans.

Plants from treated seeds were grown for 54 days in the greenhouse after which they were harvested and the green weights determined for each crock. These seedlings were not thinned upon completion of germination, so the total green weights indicate survival as well as size of the plants. The heaviest bean plants were obtained from seed exposed to carbon dioxide treatment in tap water, a result which will be corroborated in data to be discussed under "Further growth of seedlings". Bean growth was not improved by any of the trace elements tried. Best growth of oat was obtained from seed soaked in molybdate solutions. Non-aeration or carbon dioxide treatment gave the same results and both of these were better than oxygen treatment. Tomatoes were benefited by treating seeds with copper sulfate and carbon dioxide. There was no favorable effect of any of the trace elements on the growth of rice, and carbon dioxide reduced the growth somewhat.

When the average weight per plant is considered, growth of corn and tomatoes was in the same order as described for total weights, but this was not true for bean plants. Here the greatest weight per plant was from oxygen treatments, since so very few plants survived (usually only one or two per pot) that there was less competition for the limited food supply in the sand. The same could be said for oats, the germination of which was drastically reduced by copper sulfate treatment, in the presence of oxygen.

All the species tried in this experiment have been shown by other work-

ers to be benefited by one or more of the trace elements used, and some of the results may be suggestive. Whereas fairly large amounts of a major element such as phosphorus would be required for even the young stages of growth, smaller quantities of trace elements would be necessary. This fact, together with the frequent deficiencies in trace elements exhibited by many soils, warrants further investigation of the possibilities of supplying these elements by soaking the seeds in solutions containing them before planting. Such a method has been used to satisfy the boron requirements of cotton (16) and to control the molybdenum deficiency in subterranean clover (7). The extra tool for this procedure furnished by gas effects may be significant.

Toxic substances. Carbon dioxide protected wheat seeds against injury from 0.02 per cent sodium selenate during a soaking period of 24 hours, allowing 97 per cent germination as compared with 0 per cent after 24 hours' soaking in the same solution in the presence of oxygen. Carbon dioxide was also found to be strikingly effective in the prevention of injury to bean seeds by solutions of 2,4-dichlorophenoxyacetic acid (2,4-D). The possibility of conditioning seeds by presoaking in water in the presence of carbon dioxide so that they would not be affected by 2,4-D incorporated in soil was tested with negative results. Seeds so conditioned were not more resistant to 2,4-D than dry seeds of turnip, mustard, bean, oat, or wheat. However, oxygen-treated seeds were less resistant than dry seeds.

FURTHER GROWTH OF SEEDLINGS

Because of the speed and vigor of germination of bean seeds after soaking in water saturated with carbon dioxide and the delay in germination caused by air and oxygen, some experiments were designed to determine whether these effects may be carried over to the vegetative and fruiting stages of the plants produced.

For this purpose dry bean seeds were soaked for 24 hours in the laboratory in both tap and distilled water through which air, oxygen, nitrogen, or carbon dioxide was bubbled. Non-aerated and, in the case of tap water, running water controls were used in addition to the dry control. The same treatments were given to seeds which had been "conditioned" before soaking. According to Eyster (8), bean seeds may be conditioned to tolerate submergence by keeping them on filter paper wet from below for 24 hours before placing in water. Altogether, then, seeds from 24 different treatments were planted in pots in the greenhouse. Ten replicate 5-inch pots containing five seeds each for each treatment were planted. The pots were arranged on the greenhouse bench in ten blocks, each block containing one pot from each treatment arranged in a random fashion determined from tables of random numbers. The entire planting was surrounded by pots each containing five untreated seeds. After the germination was complete

the seedlings in each pot were thinned to two, the two farthest apart, regardless of size. The planting was made in the greenhouse on September 21 and the plants were harvested on October 13. Dry weights of the stems and leaves were determined for each pot. No variation in germination was found in the blocks, though there were some differences in dry weights of the seedlings. "Conditioning" the seeds removed none of the harmful effects of soaking in tap water, but increased germination somewhat in non-aerated and aerated distilled water. In general, distilled water was more harmful than tap water, for both germination and vegetative growth. Very few plants survived the oxygen treatment. The average dry weights in grams per plant for seedlings from dry seeds soaked in tap water were: carbon dioxide—1.01, running water—0.98, nitrogen—0.98, dry control—0.96, non-aerated—0.93, air—0.88, and oxygen—0.83; dry weights of plants from "conditioned" seeds in the same series and in the same order were 0.94, 0.86, 0.91, 0.95, 0.89, 0.80, and 0.51.

A second planting made in the greenhouse with an arrangement as described above included dry seeds with and without Arasan treatment before soaking in tap water with the pH adjusted to 7.55 or 3.15 and with air, carbon dioxide, or nitrogen passing through the soaking solution. Seeds soaked in non-aerated water were used as controls. After treatment for 24 hours, all seeds were planted in greenhouse pots on October 19 and were harvested on December 8 and 9 after pods had begun to set on the plants. Wet weights of stems, leaves, and pods and the number of pods were taken for each pot. A statistical analysis of the data for the total weights revealed a significant difference between the gas treatments, as a result of the higher weights of the plants from carbon dioxide-treated seeds. The only other significance was in the interaction between the Arasan treatment and the experimental plots, demonstrated by the variation in the performance of the Arasan-treated seeds in the different plots. There was a close correlation between the weights of the leaves and the pods, but the stems varied in weight. Apparently some of the increased vigor and early growth rate resulting from the carbon dioxide treatment were carried over to the maturity of the plant. It should be pointed out that adverse light conditions prevailed throughout the growing period. We have already seen how carbon dioxide-treated seeds resisted the unfavorable effects of various harmful salts in solution and it may be that the resulting plants also have more resistance to unfavorable growing conditions.

The presoaking of seeds, especially grains, under controlled conditions is claimed to hasten maturity and seed production. This process, known as vernalization or yarovization, is usually carried out at fairly low temperatures and with limited amounts of water. For details of such treatments, the reader is referred to a book entitled "Vernalization and Photoperiodism" (15). The type of soaking considered in the present paper is concerned

more with shorter periods at ordinary room temperatures with an excess of soaking solution. Even under these conditions, reports have been made of the favorable effect of soaking the seed in non-aerated water on the growth of the plants produced from the seeds.

Kidd and West (13) demonstrated the retarding effect on the growth of seedlings of *Pisum sativum* and *Hordeum* as a result of soaking the seeds prior to planting. Even six hours of soaking retarded the growth of *Phaseolus* 26 per cent, as determined by plants harvested four and one-half weeks after sowing. Soaking for various periods had a beneficial effect on the subsequent growth of wheat, oat, white mustard, and *Vicia faba*, and no apparent effect on white lupin. A progressive reduction in germination as well as in growth of the seedlings with increased time of soaking was described by Bailey (4). Albaum (2) soaked oat seeds for 20 hours in water through which oxygen-nitrogen mixtures containing 0, 2.5, 5, 10, 20, 50, and 100 per cent oxygen were bubbled. After exposure to pure oxygen the germinating seeds failed to develop normal coleoptiles, their length being inhibited markedly.

Andrews and Beals (3), and Sawhney (18) accelerated the speed of germination of corn by soaking in water for 8 to 12 hours before planting. No claims were made in either of these reports for augmented seedling growth as a result of the seed soaking, but Tincker (19) found that soaking oat grains in water accelerated seedling growth and concluded that a definite correlation existed between the vigor of germination and the rate of subsequent growth. Some of the good effects on seedling growth of pre-soaking bean seeds in water as described above and the advantage of pre-soaking corn seeds in water over the dry controls to be described below are no doubt closely related to the vernalization responses shown by other workers. Further investigation may show that the vernalization process may be effected simply by soaking seeds with controlled gas supply without the necessity for the exact control or the longer time required under the recommendations as they appear in the literature at present. Some such simple procedure might remove the variation from the responses reported by various authors.

Because of the importance of incorporating fertilizers with seeds through the soaking process and the failure of many such trials due to injury to the seeds by the salts, seedlings produced in the greenhouse from the plantings shown in Tables IV and V were kept for observation. Due to the lack of nutrients in the soil used, several different factors may have been limiting to growth, but some interesting differences were observed, and are shown in Figures 3, 4, and 5.

In Figure 3, which pictures corn seedlings 19 days after planting seeds which had been soaked for four hours, it will be seen (A) that soaking in water resulted in larger seedlings than appeared from the dry seeds. This



FIGURE 3. Effect on seedling growth of soaking corn seeds for 4 hours. (1) Seeds planted dry; (2, 3, and 4) seeds soaked in solutions with no aeration or aerated with oxygen or carbon dioxide. Solutions: (A) tap water, (B) 1.0 M KNO_3 , or (C) 1.0 M K_3PO_4 .

may have been due to the earlier beginning of germination as a result of the four hours' soaking since plants from dry seeds seemed to catch up with those soaked in water at a later time. In Figure 3, one can also compare the effect of non-aerated solutions with those supplied with oxygen or carbon dioxide during the soaking process. At this time, seedlings from carbon dioxide treatment showed poorer growth than others, especially those of 1.0 M KNO_3 , in either non-aerated or oxygenated solutions. The



FIGURE 4. Effect on seedling growth of soaking corn 24 hours (A) or oat 4 hours (B) in solutions aerated with carbon dioxide. Left to right: dry control, tap water, 0.5 M KNO_3 , 1.0 M KNO_3 , 1.0 M K_3PO_4 . Planted September 20; photographed November 1.

destruction of seeds in non-aerated and oxygenated 1.0 M potassium phosphate and the reduced germination after soaking in carbonated solutions of this salt are also shown.

On the day these photographs were taken, all corn plants both in these and other pots, were thinned to five per pot, in order to equalize competition for space and soil nutrients. Later growth is shown in Figures 4 and 5. Figure 4 A shows corn seedlings six weeks after planting seeds presoaked in solutions supplied with carbon dioxide. Here it is evident that the seeds soaked in the phosphate retained enough of the salt to permit increased growth over those not supplied with this salt. Not only were the plants taller, but their stems were very much larger than the others. The wet and dry weights of the stems and leaves of plants grown from seeds soaked in 1.0 M K_3PO_4 were more than twice that of those from any other treatment

75 days after planting. The same effect to a lesser degree was noted for oats.

In Figure 5, which shows corn plants nine days older than those in Figure 4 A, the continued favorable growth of the phosphate-treated seedlings is noted. Also, another effect which had not been so apparent up to that time had become evident, namely, the relatively good growth of seeds oxygenated while soaking in solutions of KNO_3 . This may mean that oxy-

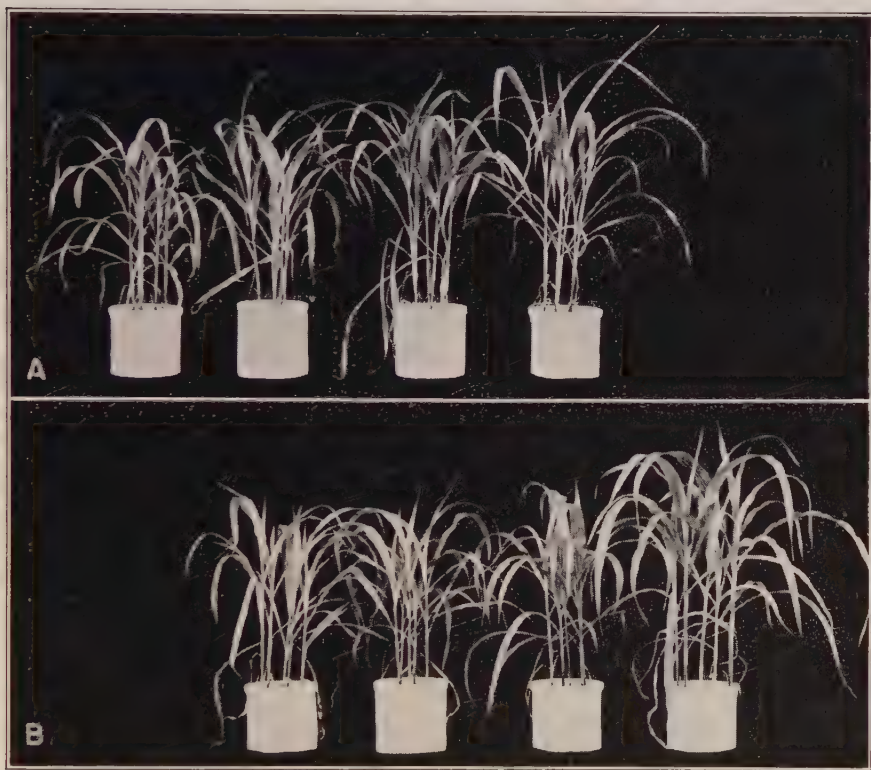


FIGURE 5. Effect on seedling growth of soaking corn for 4 hours. (A) Left to right: dry control; and soaked and aerated with oxygen in tap water, 0.5 and 1.0 M KNO_3 ; (B) (Dry control as above.) Left to right: soaked and aerated with carbon dioxide in tap water; 0.5 and 1.0 M KNO_3 ; and 1.0 M K_3PO_4 . Planted September 21; photographed November 10.

gen facilitates the entry of the salts and if the concentration is not sufficient to kill the embryos of the seeds, the nutrient is retained for the use of the seedlings. Carbon dioxide, on the other hand, retards the entry of the harmful solutions so that they do not always reach the embryo, but enough of the salts may be retained in the seed coats or other parts of the seeds, to act as fertilizers after the seed has germinated. Under the conditions of the present tests, some of the harmful phosphate did affect the embryos as

evidenced by the reduced germination after such treatment in the presence of carbon dioxide. Similar effects are shown for oats in Figure 4 B. It appears that this problem should be approached from two directions: one, the favorable effect of carbon dioxide in preventing injury when seeds are soaked in strong salt solutions or in weaker solutions for longer periods of time, and the other, the action of oxygen in facilitating the uptake of non-toxic salt solutions, thus permitting a greater accumulation of the nutrient without subsequent injury to the embryos.

Eighty-two days after seeds treated as indicated in Table VI were planted in the greenhouse they were harvested and the wet and dry weights of the stems and leaves were recorded. Plants were thinned to five plants per crock one week after planting. In the over-all picture for corn growth, soaking in 0.5 M K_3PO_4 with oxygen or nitrogen gave a big increase in plant size. However, the number of seeds germinating and plants surviving was greatly reduced. Except for this instance, there was probably no significant favorable effect of soaking in salt solutions. The failure of CO_2 treatment to result in increased size of resulting plants in 0.5 M K_3PO_4 is in contrast to the striking increased growth from the same gas treatment in a stronger solution (1 M) as reported above. Similarly, the favorable effect of the oxygen and nitrogen treatments with the weaker solution is in contrast to the lack of survival of seeds so treated with the stronger solution (Table IV). Though less marked than for corn, oat seed germination and subsequent growth of the seedlings followed the same pattern (Table VI).

A new greenhouse test for growth of corn plants after soaking seeds was performed using seeds soaked in tap water, 1 M and 4 M K_2HPO_4 , and 1 M and 4 M K_3PO_4 with different gases. Four hours' soaking was used for all solutions except K_3PO_4 where a thirty-minute period was used since four hours' soaking had been previously demonstrated to be very injurious to germination. Sixty-two seeds were soaked in 50 ml. solution. At the end of the soaking period all seeds floated in the 4 M solutions. Dry seeds were also used as a control of the soaking effects. Replicates of six lots of ten seeds each were sown after treatment in two-gallon glazed crocks containing a very poor sod soil with no fertilizer of any kind. After soaking and draining off soaking solution and washing for 15 seconds in a sieve under running tap water, each lot of seed was placed in a filter paper bag in a Petri dish until all treatments were complete when they were planted. Thus the seeds were not allowed to dry out before planting. After planting, the pots were arranged in a split block design in the greenhouse. Germination records were taken. Eight days after planting the seedlings were thinned to two plants per crock. Records were kept of the date of flowering and wet and dry weights of stems and leaves were taken 82 days after planting.

Time of flowering, apparently, was not influenced materially by the

treatments. On the other hand, the number of plants failing to flower by the termination of the experiment may be indicative. For example, for 1 M K_3PO_4 treatment, plants in 15 out of 30 crocks, or one-half, failed to flower, while only 5 from 1 M K_2HPO_4 treatment, and 6 from tap H_2O treatment did not produce flowers showing a correlation with plant weights to be given below. The other three treatments, dry seeds, 4 M K_2HPO_4 and 4 M K_3PO_4 resulted in failures in 9, 10, and 11 pots respectively.

Dry weights of the tops of the plants (stems and leaves) when harvested are shown in Table VIII, where each number represents the total of two plants in each of six crocks from the six rows of the experiment for each treatment. An analysis of variance of these data revealed a significant difference between treatments with the different solutions, but no significance for gases or rows and no significant interactions. The highest plant weights

TABLE VIII

SEEDLING PRODUCTION IN THE GREENHOUSE AND DRY WEIGHTS OF PLANTS PRODUCED AFTER SOAKING CORN SEEDS IN THE LABORATORY

Soaking solution*	Effect of different gases supplied during soaking									
	Germination, %					Dry wt. of tops in g.				
	None	Air	O ₂	N ₂	CO ₂	None	Air	O ₂	N ₂	CO ₂
None (dry)	100	98	98	97	100	71	83	66	72	62
Tap H ₂ O	98	98	100	100	100	72	74	92	77	74
1 M K ₂ HPO ₄	83	95	92	93	98	83	89	76	87	87
4 M K ₂ HPO ₄	98	98	95	93	90	68	54	73	81	65
1 M K ₃ PO ₄	93	87	88	87	93	43	44	46	61	70
4 M K ₃ PO ₄	75	77	77	87	88	72	46	43	46	88

* Four-hour soaking for all except 1 M K_3PO_4 where 0.5 hour was used.

were from seeds treated with 1 M K_2HPO_4 . These were the only plants with greater weights than those soaked in tap water, which were significantly better than those from the dry seeds. Soaking itself, then, was beneficial but a solution of 1 M K_2HPO_4 gave additional benefits. Other solutions produced seedlings of less weight than the tap water or the dry controls. It will be noted that the favorable effects of 1 M K_3PO_4 reported above were not evidenced here. This may be due to a shorter period of soaking or washing of the seed before planting. The data on dry weight are in substantial agreement with the green weight and height of plants.

A field planting was made on May 16, 1951, in very poor soil. Seeds were pretreated as indicated in Table IX. Pretreatments which had shown some promise from the standpoint of the growth of the plants and which did not drastically reduce the stand were selected. Also, a comparison of potassium and sodium phosphates was made. One 4 M solution was included. The field plot was arranged in incomplete blocks as was the green-

house planting described above, with the exception that each replication was represented by only one plant, instead of two, thinned on June 8, 1951 from the original planting of ten seeds. The heights of the plants were taken on August 3, and the grain was harvested on October 1. The grain was shelled from the ear and the dry weights taken. Data on height of plant, weight of grain, and percentage germination are summarized in Table IX.

TABLE IX
FIELD PERFORMANCE OF CORN PLANTS FROM SEEDS SOAKED IN VARIOUS SOLUTIONS

Effect on plants	Gas supplied during soaking	Totals for 6 rows after soaking in solutions					
		Tap H ₂ O (4)*	K ₂ HPO ₄		K ₃ PO ₄ 1 M (0.5)*	Na ₂ HPO ₄ 1 M (4)*	Na ₃ PO ₄ 0.5 M (0.5)*
			1 M (4)*	4 M (0.5)*			
Height, in.	None	458	424	479	363	391	414
	Air	472	433	—	385	489	412
	O ₂	457	440	447	325	448	424
	N ₂	491	425	448	396	456	427
	CO ₂	411	399	450	426	441	458
Dry wt. of grain, g.	None	289	571	456	557	326	620
	Air	581	428	—	589	923	628
	O ₂	508	557	439	288	285	771
	N ₂	498	439	475	439	544	173
	CO ₂	609	505	574	746	293	759
No. of grains	None	1417	2367	2339	2908	1547	3268
	Air	3052	1956	—	2898	4373	2950
	O ₂	2644	2976	2277	1511	1746	3149
	N ₂	2587	1522	1977	1981	3035	1017
	CO ₂	3589	2125	2773	3456	1692	3258
Per cent germination	None	98	90	92	53	97	75
	Air	98	85	—	57	88	85
	O ₂	97	90	98	43	88	73
	N ₂	95	88	93	57	97	77
	CO ₂	97	88	90	85	98	93

* Numbers in parentheses indicate hours of soaking.

The most significant reduction in germination took place following the soaking in 1 M K₃PO₄, where carbon dioxide permitted subsequent germination of 85 per cent but only 43 to 57 per cent germinated after treatment with other gases. Some reduction in stand followed soaking in 0.5 M Na₃PO₄ with all gases except carbon dioxide. None of the other soaking treatments affected germination in the field.

When the data on height of the plants are considered there is a significant difference in the plants from seeds treated with the different solutions, with those from seeds soaked in 1 M K₃PO₄ showing poorest growth. This may be accounted for, at least in part, by the delayed germination of these seeds, the germination of which was reduced by the treatment. The gases

supplied during the soaking did not affect the heights of the plants. The rows differed significantly from each other. The performance of plants from dry seeds approximated that of those from seeds soaked in non-aerated tap water.

In total grams of grain produced a significant difference can be shown to exist between the performance in the various rows. However, because of the failure of many plants to produce any grain, it is not possible to draw definite conclusions as to treatment effects. Totals for the various lots are shown in Table IX and certain of them may be suggestive. Production may have been stimulated by 0.5 M Na_3PO_4 and 1 M K_3PO_4 . Also, there undoubtedly was interaction between the solutions and the gases supplied during soaking, but not consistently in one direction. For example, in tap water soaking, non-aeration was most injurious and carbon dioxide least harmful. Carbon dioxide also permitted the best performance of plants from seeds soaked in 4 M K_2HPO_4 and 1 M K_3PO_4 , and creditable performance was noted for seeds supplied with this gas during soaking in 1 M K_2HPO_4 and 0.5 M Na_3PO_4 . In contrast, solutions of 1 M Na_2HPO_4 were without favorable effect. The superior performance of plants with oxygen supplied during soaking in Na_3PO_4 and K_2HPO_4 should be pointed out. Differences in the weights of grain produced were due in general to increase in the number of grains though there was some variation in the individual grain weights.

In view of the extra amount of potassium made available by using potassium instead of sodium phosphates it might be commercially advisable to use the former. However, corn seeds contain rather large quantities of potassium so its addition may be effective only with certain seeds and certain soils.

It is recognized that in types of deficient soils used for both greenhouse and field tests, many factors besides the ones considered may be limiting to growth. Similar tests need to be conducted using soils of different fertilities.

The incorporation of needed fertilizer with the seeds before sowing has been the goal of many workers. Gleisberg (10) reported in 1924 the increase of the vegetative growth of radish 364 per cent by treating the seeds with a solution containing 15 per cent magnesium chloride and 15 per cent magnesium sulfate. Solutions of manganese and magnesium salts as seed treatments for rice gave no increased growth of the seedlings as claimed by Popoff (Becker, 6). On the other hand, Abichandani and Ramiah (1) reported in 1951 that rice seeds soaked in one-third of their weight in nutrient solutions for 24 hours and sown in phosphate-deficient soil gave increased yields. Roberts (17) described the prevention of mineral deficiency by soaking wheat, barley, oat, and rye seeds in nutrient solutions containing 1 ml. per 3 gm. After soaking, the seeds were dried to their original weights in a

current of warm air not above 22° C. and then sown in soil deficient in either phosphorus or manganese, which were the elements tested. The results of greatest practical importance were obtained by soaking oat seeds in 1 M tribasic potassium phosphate which brought about a 46 per cent increase in yield on phosphate-deficient soil. There was also an increase in the weight of straw. The concentrations of chemicals used by Roberts were of the order of 1 M. Injury to the seed was prevented by limiting the amount of chemical solution used in soaking. In the present tests, absorption of solution and thus both seed injury and any fertilizer effects were controlled by passing gases through the solutions during the soaking period. It is conceivable that a practical application of the whole idea of incorporating fertilizer with the seed by presoaking could be worked out, using nitrogen to facilitate the entrance of weak solutions or carbon dioxide to withhold too great penetration of stronger solutions, at the same time permitting the deposition of effective amounts of the fertilizer in the parts of the grain surrounding the embryo, and hence not injuring the embryo. This means the use of solutions much weaker than those described by Gericke in his patent (9). His method is based on the use of solutions too concentrated to penetrate into the seed, and stipulates that the concentration should be sufficiently strong to cause the seed to float in the solution.

It should be kept in mind that the fertilizer effects described in this paper are separate and distinct from the effects of small amounts of stimulating substances such as hormone-like chemicals, thiourea, etc., and from insecticidal or fungicidal effects on growth.

Field tests should be conducted in which soils of different types and fertility should be planted with seeds soaked in excess and in limited amounts of solutions supplied with air, nitrogen, or carbon dioxide during the soaking process.

The key to the effects of the various gases may be found in the control of water absorption. Evidence of this has been given previously in a report from this laboratory (5). Additional evidence has been presented in this paper. However, it is still not possible to attribute all effects to moisture absorption only. Further work, especially on the nature and amounts of enzymes involved, is needed.

SUMMARY

Repeated experiments have shown the deleterious effect of oxygen and the protective effect of carbon dioxide when these gases are passed through solutions in which bean, corn, and oat seeds are soaking. Non-aerated solutions or those through which air or nitrogen are passed are less injurious than oxygenated solutions, but do not offer the protection afforded by carbon dioxide.

Excessive absorption of water seems to be a factor in this deterioration. Polyvinylpyrrolidone in 5 and 10 per cent solutions retarded the absorption of water by seeds and reduced soaking injury in the presence of oxygen, but had no effect in the presence of carbon dioxide. Use of a vacuum or surface-active agents to facilitate the entrance of water into the seeds was without effect on subsequent germination, which depended on the length of the soaking period.

Rice seeds which germinate normally under water were not injured by the presence of oxygen in the water, but their water absorption and germination were delayed by treatment with carbon dioxide during soaking.

Injury of bean, corn, oat, and wheat seed by soaking in salt solutions containing major and minor elements was prevented by supplying carbon dioxide during the soaking process. A supply of oxygen during soaking was consistently harmful to bean, corn, oat, and wheat seeds under the same soaking conditions. Measurements of the amount of phosphorus and potassium absorbed, germination ability, and growth of plants produced after soaking in various solutions with different gas supply indicated the possible incorporation of fertilizers by soaking seeds before planting. Deficient trace elements may also be supplied in this manner.

Carbon dioxide not only protected seeds against injury by soaking in water or nutritive salt solutions, but also prevented toxicity to embryos by selenium salts and 2,4-dichlorophenoxyacetic acid, and increased the resistance of corn seeds to low temperature.

Increased growth of bean seeds soaked in carbonated water prior to planting was demonstrated.

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RESPONSE OF TOMATO PLANTS TO TREATMENT WITH 2,4-DICHLOROPHENOXYACETIC ACID IN COMBINA- TION WITH INDOLEACETIC ACID AND CERTAIN OTHER COMPOUNDS

A. E. HITCHCOCK AND P. W. ZIMMERMAN

INTRODUCTION

Many interactions are known to occur when small amounts of chemicals are applied in combination with a growth regulant to plants. Various explanations proposed for the probable role of auxins and auxin inhibitors in some of the important metabolic processes in plants have been reviewed by Larsen (7), Mitchell (8), Bonner and Bandurski (1), and van Overbeek (9). The inhibitive and additive effects induced by substances of diverse chemical structure when applied in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) to tomato plants were described briefly in previous reports (3, 5, 12). These effects were based on changes in the magnitude of growth and formative responses as compared with those induced by an equivalent dose of 2,4-D. Changes in the action of 2,4-D brought about by indoleacetic acid (IA) were essentially the same as those induced by substances which are not active for cell elongation, such as 1-naphthoxyacetic acid (1-NOA), 2-naphthaleneacetic acid (2-NA), trans-cinnamic acid, monochloroacetic acid, iodoacetic acid, and sodium fluoride. In general, additive effects were obtained with lower doses of 2,4-D than those involving inhibitive effects.

Changes in the action of 2,4-D and in the action of IA and several other growth regulators were brought about by 1-NOA. These results show that the structural requirements of an activator or inhibitor are not limited to one type. The responses resulting from the combination treatments with IA and 2,4-D were the same as those induced by a correspondingly higher or lower dose of 2,4-D, even though IA and also other substances were inactive for one or more of the responses induced by 2,4-D, or were inactive at the dosage levels used.

Although 2,4-D is not known to occur in healthy plants, under the influence of certain viruses some species of plants develop modified leaves and exhibit other symptoms similar to those induced by 2,4-D, *p*-chlorophenoxyacetic acid, and 2-NOA (2). Such effects were intensified or depressed when bean and aster plants artificially infected with Southern Bean Mosaic virus were then treated with one of the aforementioned growth regulants. Since the concentration of virus in the host was not correlated

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with the severity of symptoms, and the growth regulant did not change the activity of the virus *in vitro*, it appears that the additive and inhibitive effects were not caused directly by virus molecules, but rather by a reaction product of virus activity (2, p. 827). The isolation of a non-infectious leaf-modifying substance from tomato plants infected with tobacco mosaic (6) lends support to the concept that there exists in plants a mechanism capable of producing leaf-modifying growth regulants. The results obtained with Southern Bean Mosaic and growth regulants are of special interest since they showed that the two components of the combination treatment affected different processes independently in some cases and the same processes in other cases. In some respects the findings are similar to those reported in the present paper.

Results of the present tests indicate that the capacity of 2,4-D to modify leaves is an inherent property of the 2,4-D molecule which appears to act on a growth-regulating mechanism independently as well as in conjunction with the mechanism controlling cell elongation. The capacity of a growth regulator to modify leaves and other organs is not a property either of IA or of the naturally-occurring auxins. Since 2,4-D and IA do not bring about the same type of growth regulation, it follows that, if present in healthy plants, 2,4-D could not substitute for naturally-occurring auxins of the IA type.

This report is concerned primarily with the additive and inhibitive action of IA when applied in combination with 2,4-D to tomato plants. A brief description of the results obtained with other combination treatments is also presented.

MATERIALS AND METHODS

Growth regulators. All growth regulants were formulated as triethanolamine salts. The parent 2,4-D acid had a melting point of 140° C. and a chlorine content of 32.3 per cent. The triethanolamine salt of this acid was equivalent in activity to a commercial formulation of mixed amine salts (Dow "40") used previously (5).

The concentrations of 2,4-D used in most tests ranged from 0.001 to 0.1 per cent which, in the case of the 0.01 ml. application, represented doses of 0.1 to 107 2,4-D per plant. In tests involving the removal of treated leaves, concentrations up to 40 per cent were used. Concentrations of IA ranged from 0.01 to 10 per cent.

Treatment of plants. The selection and treatment of Bonny Best tomato (*Lycopersicon esculentum* Mill.) plants were the same as previously described (5). In the principal series of tests involving the use of IA and 2,4-D, 0.001 to 0.01 ml. of test solution was applied by means of a pipette. The solution was spread from the point of contact of the pipette near the basal part of the midrib of a leaflet toward the apex in order to dislodge all of the measured volume of solution clinging to the point of the pipette.

An improved method consisted of applying test solutions by means of fused platinum true circle loops calibrated to deliver 0.001 or 0.01 ml. and having inside diameters of 1.45 mm. and 4 mm. respectively. Each of these two methods of applying test solutions gave consistent and reproducible results, and the responses induced were approximately equivalent. The loop was readily cleaned by first dipping into water and then pressing it between clean pieces of paper towel. Drying the loop over the flame of a cigarette lighter was tried, but this precaution was abandoned when it was found that no detectable contamination occurred when the loop was dried with a piece of paper towel. Lack of contamination was determined by treating control plants with distilled water intermittently between applications of 2,4-D by means of the same loop. The improved method appears to offer the least opportunity for unwanted contamination of any method used in this laboratory for the application of growth-regulator solutions to plants. Another method of treatment consisted of applying mixtures of IA and 2,4-D to the soil or applying one substance to the soil and the other substance to the foliage.

When only one leaflet was treated, the terminal one was used, and in the case of treating two leaflets, those next to the terminal were used. Results varied according to the number and position of the treated leaflets, but treatment of a leaflet occupying the same morphological position gave consistent results in different tests. Treated plants in some cases were subjected to the gravity stimulus and oriented so that the treated leaf was either on the upper or lower side of the stem.

Evaluation of responses. Curvature of stems was measured with a protractor and expressed in degrees. Increase in height of the treated plants over that of controls was measured in millimeters. Modification of leaves and proliferation of stems were estimated on the basis of a magnitude of response scale ranging from 1 (trace) to 4 (maximum). The estimated magnitudes of modification and proliferation on three plants were multiplied by a factor of 5 or 6. For example, if the estimated magnitudes of proliferation on three plants were 2, 2, and 3 respectively, the total of 7 was multiplied by 5 to give a value of 35. This was done in order that the numerical values representing the maximum amount of modification and proliferation would be of about the same order of magnitude as for the maximum height increase in terms of millimeters. Since the growth and development of tomato leaves were associated with an increase in height of the plant, growth and increase in height are used synonymously in this report.

Responses on treated plants were evaluated at various intervals up to the time the test was terminated, which was generally after 10 to 14 days. In addition to measuring or otherwise evaluating individual responses, the plants were ranked according to the total effect of the treatment (5). Ranks were assigned on the basis of the relative dose of 2,4-D required to induce

the responses being evaluated. Highest ranks were assigned to plants exhibiting the most pronounced responses which, in the case of 2,4-D treatments, would mean to the plants receiving the highest dose of 2,4-D. Plants treated with IA in combination with 2,4-D would therefore receive the same or different ranks accordingly as the effect of IA was additive or inhibitive with respect to the responses induced by an equivalent dose of 2,4-D.

RESULTS

Leaf applications of 0.01 ml. Results in Table I show that the mixed amine and the triethanolamine salt formulations of 2,4-D are of about equal activity and of lower activity than the acid when ranks for all responses

TABLE I

COMPARATIVE EFFECTIVENESS OF DIFFERENT FORMULATIONS OF 2,4-D WHEN 0.01 ML. OF THE TEST SOLUTION WAS APPLIED WITH AND WITHOUT A SPREADER TO ONE LEAFLET ON TOMATO PLANTS

Formulation of 2,4-D	Nekal NS spreader 0.5%	Magnitude of response according to γ 2,4-D per plant							
		Stem curva- ture degrees		Modification of leaves		Proliferation of stems		Ranks* for all responses	
		I	4	I	4	I	4	I	4
Acid	o	o	30	28	52	o	16	3.5	8.5
	+	o	40	36	36	o	36	6	12
Triethanolamine salt	o	o	40	24	40	o	16	1.5	8.5
	+	o	42	28	48	o	24	3.5	10
Dow "40" (mixed amine salts)	o	o	38	24	36	o	8	1.5	7
	+	o	38	32	52	o	28	5	11

* Highest rank represents most effective formulation.

are considered. All three formulations were slightly more effective when used with 0.5 per cent Nekal NS spreader (General Dyestuff Corp.). The relative degree of leaf modification was used as the principal basis for ranking plants treated with 17 2,4-D. Both proliferation and modification were used as the basis for ranking plants treated with 47 2,4-D, with most emphasis being placed on the relative amount of proliferation. As will be shown subsequently, the degree of leaf modification increases to a maximum and then decreases to a zero value, whereas the amount of proliferation increases or remains at a high value throughout the sub-lethal range of dosage.

Treatment of one leaflet on the third or fourth leaf with IA and one leaflet on the other leaf with 2,4-D generally resulted in additive effects. Applying IA and 2,4-D to different leaflets on the same leaf resulted in either additive or inhibitive effects depending upon the relative number of

leaflets treated, the dosage level of each compound, and the morphological position of the treated leaflets (Table II). As the distance between the treated areas was increased, the additive effects became more pronounced. In view of these differences, it is evident that leaflets occupying the same morphological position should be treated in comparative tests.

The relationships between the dosage-response curves for 2,4-D in the

TABLE II

ADDITIVE AND INHIBITIVE EFFECTS INDUCED BY IA WHEN APPLIED IN COMBINATION WITH 2,4-D TO DIFFERENT LEAFLETS ON THE FOURTH LEAF OF TOMATO PLANTS

Dose per leaflet and position of treated leaflets		Height increase over controls, cm.	All responses, rank*	Relative effect of IA**
100γ IA	10γ 2,4-D			
Terminal	None	2	5	
First pair	None	2	5	
Second pair	None	1	5	
None	Terminal	9	15	
None	First pair	-2	37	
None	Second pair	4	29	
One, first pair	One, first pair	10	18	
First pair	Terminal	3	29	Additive
Second pair	Terminal	7	15	None
Second pair	First pair	1	22	Inhibitive
First pair	Second pair	1	26	Inhibitive
One each of first and second pair	One each of first and second pair	-1	35	
Terminal	First pair	2	30	Inhibitive
Terminal	Second pair	-1	33	Additive

* Highest rank represents greatest response.

** As compared with similar leaflets treated only with 2,4-D.

range 1 to 10γ and for mixtures of 100γ IA and 1 to 10γ 2,4-D are shown in Figure 1. The activity of mixtures of IA and 2,4-D was considerably lower than equivalent quantities of 2,4-D used alone, and consequently in this case IA functioned as an inhibitor of 2,4-D. For example, the mixture containing 8γ 2,4-D induced responses about equivalent to those induced by 4γ 2,4-D alone, so that in this case IA reduced the action of 2,4-D by approximately 50 per cent. The mixture containing 10γ 2,4-D induced responses much the same as those induced by 10γ 2,4-D, indicating that, in this particular test, 100γ IA was not a sufficient quantity to inhibit the action of 10γ 2,4-D. Since modification of leaves is dependent upon growth, the shapes of the growth and modification curves are similar, and they are inverse to the shape of the curve for proliferation when the dose of 2,4-D is 1 to 10γ as shown in Figure 1. Thus, as growth and modification increase, proliferation decreases, and, conversely, an increase in proliferation is as-

sociated with a decrease in growth and modification. Green weights of the three youngest leaves taken from these plants (totals 2, 3, 4, Table III) reflect the same relative differences as are shown for growth and modification in Figure 1. The green weights were higher for the three youngest leaves on plants treated with mixtures containing 100 γ IA and 6 to 8 γ

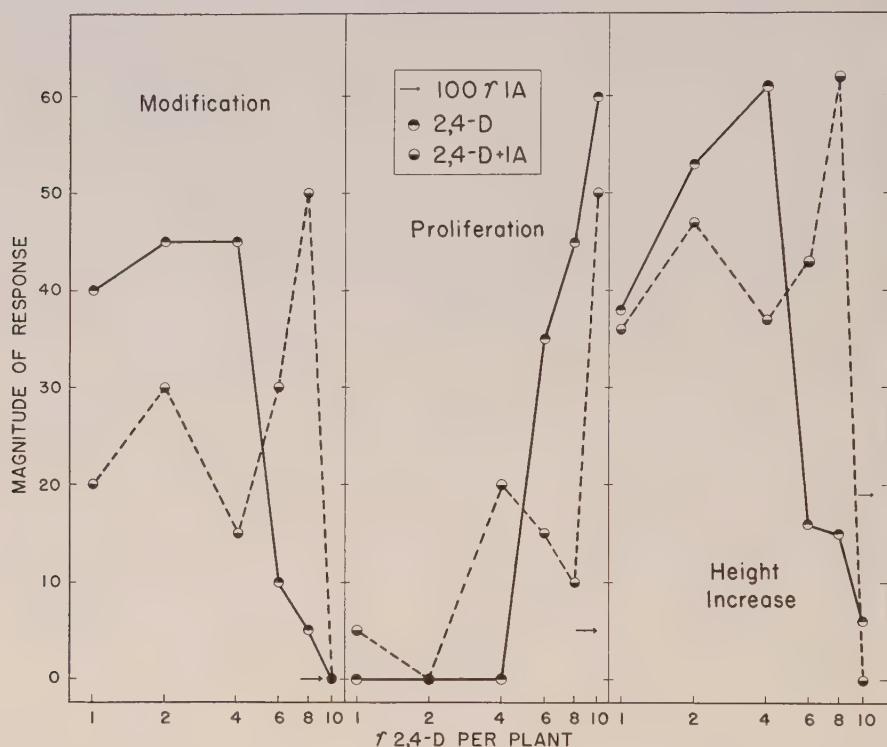


FIGURE 1. Inhibitive action of IA resulting from treatment of tomato plants with 0.01 ml. applications of mixtures containing 100 γ IA and different quantities of 2,4-D. Responses evaluated 9 days after treatment.

2,4-D as compared with corresponding leaves on plants treated with 6 to 8 γ 2,4-D.

Additional examples of inhibitive effects involving the use of 0.1 to 10 γ 2,4-D are shown in Tables IV and V. Additive effects generally resulted from treatments with 1 γ or less of 2,4-D. The quantity of 2,4-D appeared to be of greater importance than the ratio of IA to 2,4-D in determining whether the effect of IA was additive or inhibitive. Evaluation of treatments involving 0.1 to 1 γ 2,4-D or less and less than 100 γ IA were usually based on responses other than proliferation since at this lower dos-

age level, 0.01 ml. applications of the test solution induced little or no proliferation (Fig. 1 and Table IV).

Removal of the treated leaf at various time intervals after treatment ranging from 1 minute to 24 hours resulted in plant responses much the same as those induced by lower doses of 2,4-D applied to plants that did

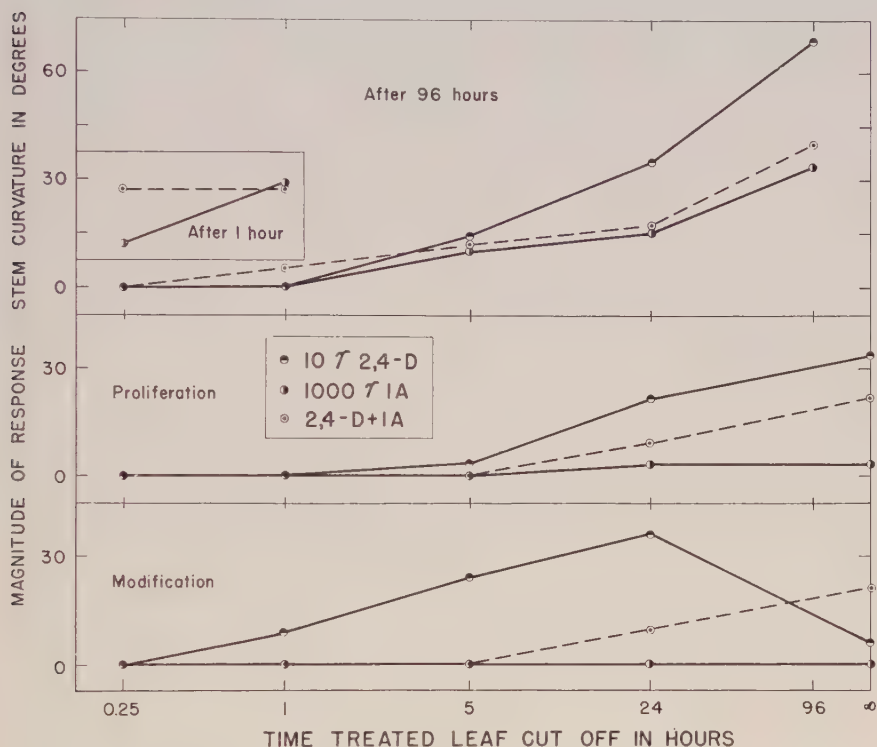


FIGURE 2. Effect of removing treated leaf at various time intervals after treatment. Stem curvature measured after 1 and 96 hours, and proliferation and modification after 15 days.

not have the treated leaf cut off. Responses evaluated 15 days after treatment are shown in Figure 2 and the appearance of some of the plants is illustrated in Figure 3. The results show that the maximum effects of the treatment were not reached in 24 hours, since the responses were of a greater magnitude on intact treated plants than on similar plants which had the treated leaf removed at the end of 24 hours. Since there appeared to be no preferential effect of IA associated with removal of the treated leaf, the counteraction of 2,4-D by IA probably occurred in the treated leaf and not after 2,4-D was translocated to the stem.

When leaves treated with 1000⁻⁷ IA or with mixtures of 1000⁻⁷ IA and



FIGURE 3. Effect of removing treated leaf at various time intervals after treatment: (left to right) not removed, removed after 24 and 5 hours respectively. Appearance of plants 13 days after treatment with 0.01 ml. applications of (A) 1000 γ IA, (B) 10 γ 2,4-D, and (C) mixtures containing 1000 γ IA and 10 γ 2,4-D.

10 γ 2,4-D were removed 15 minutes after treatment, noticeable curvature of stems occurred in one hour (Fig. 2). The mixture induced a curvature of 27° which is greater than that induced by IA (12°) and equivalent to the curvature induced by IA when the treated leaf was left on for one hour. In contrast, the leaf treated with 2,4-D had to remain on the plant in excess of one hour in order to induce stem curvature. The initial additive

TABLE III

AVERAGE GREEN WEIGHT IN GRAMS OF LEAVES ABOVE THE TREATED FOURTH LEAF OF TOMATO PLANTS 10 DAYS AFTER TREATMENT

γ 2,4-D per plant	2,4-D					2,4-D+100 γ IA				
	Leaf position above treated fourth leaf									
	1	2	3	4	Totals 2, 3, 4	1	2	3	4	Totals 2, 3, 4
0	56	39	19	7	65	65	47	22	13	82
1	67	37	14	5	56	52	33	15	6	54
2	47	27	14	7	48	54	29	15	6	50
4	57	31	13	7	51	51	26	8	3	37
6	38	14	1	0	15	47	24	8	4	36
8	52	15	3	0	18	48	21	7	3	31
10	35	13	1	0	14	28	5	0	0	5

effect on stem curvature in this case appeared to involve an amount of 2,4-D less than the threshold value. Measurements at the end of 96 hours showed that recovery of the stems to the vertical position had occurred on plants subjected to treatment with either IA or 2,4-D alone for one hour or less, but not in the case of plants treated with the mixture for one hour. Failure of the stems to recover completely on plants subjected to treatment for one hour with the mixture is indicative of a slight additive effect, but the absence of modification is definitely an inhibitive effect. Plants subjected to treatment for a period of five hours or longer exhibited an increasing magnitude of stem curvature with increasing periods of treatment up to 96 hours. The inhibitive effect of IA on 2,4-D was pronounced for treatment periods of 24 and 96 hours (Fig. 2). The direction of stem curvature was always away from the treated leaf on plants receiving 10 γ 2,4-D or less.

Measurements of stem curvatures (Fig. 2) show that additive effects were obtained at high dosage levels of IA (1000 γ) and 2,4-D (100 γ) for short periods of treatment, followed by removal of the treated leaf and inhibitive effects with longer periods. These results are similar to those obtained with intact plants in showing additive effects at lower dosage levels of 2,4-D (1 γ or less) and inhibitive effects at higher dosage levels (Tables IV and V). With doses of 10 γ 2,4-D or higher, removal of the en-

TABLE IV

EFFECT OF TREATING ONE LEAFLET ON A TOMATO PLANT WITH MIXTURES
CONTAINING DIFFERENT QUANTITIES OF IA AND 2,4-D

γ per leaflet		Magnitude of response after 10 days		Ranks* for all responses 17 days	Relative effect of IA
2,4-D	IA	Modification	Proliferation		
0	1	0	0	11	
	10	0	0	11	
	100	0	24**	21	
	1000	0	20**	32	
0.1	0	12	0	29	None Inhibitive Additive
	0.9	16	0	29	
	9.9	4	4**	11	
	99.9	24	12**	35	
1	0	36	0	42	None Additive Inhibitive
	9	36	0	42	
	99	40	16**	45	
	999	28	24**	39	
10	0	12	40	57	Inhibitive Inhibitive
	90	24	28	55	
	990	32	16	46	

* Averages for three plants based on a total of 63 ranks in which the highest rank represents the most pronounced responses.

** Proliferation of the IA-induced type associated with rooting.

TABLE V

RELATIVE MAGNITUDE OF RESPONSES INDUCED BY DIFFERENT QUANTITIES OF IA AND
2,4-D APPLIED BY DIFFERENT METHODS TO THE FOURTH LEAF OF TOMATO PLANTS.
AVERAGE RANKS FOR THREE PLANTS ARE BASED ON
ALL RESPONSES EVALUATED AFTER 19 DAYS

γ 2,4-D per leaflet	Method of treatment and γ IA applied per leaflet										
	Each substance on separate leaflets				Mixture on one leaflet			Mixture on two leaflets			
	0	10	100	1000	10	100	1000	0	10	100	1000
Test 1											
0	—	8*	8	—	—	—	—	—	8	8	—
0.1	20	28	26	—	23	11	—	35	34	22	—
1	45	46	49	—	53	36	—	51	54	46	—
10	75	76	70	—	70	62	—	80	81	68	—
Test 2											
0	—	—	21	21	—	—	—	—	—	17	21
0.1	2	—	17	62	—	8	35	5	—	21	23
1	25	—	44	51	—	49	47	53	—	57	51
10	69	—	73	78	—	66	60	83	—	79	60

* Highest rank represents greatest response.

tire leaf 1 to 15 minutes after treatment caused more pronounced responses than removal of the treated terminal leaflet.

Treatment of one leaflet with 10 to 327 2,4-D induced proliferation on one or two internodes above the treated leaf, whereas 10007 IA or less did not. Mixtures containing 10007 IA or less did not induce proliferation on internodes above the treated leaf. This inhibitive effect together with a significant reduction of proliferation on the two internodes below the treated leaf are shown in Table VI. However, the same mixture of IA and 2,4-D induced a significantly higher magnitude of proliferation on the internode

TABLE VI

RELATION BETWEEN RELATIVE AMOUNT OF PROLIFERATION ON STEMS OF THREE TOMATO SEEDLINGS AND DOSE OF IA REPRESENTED BY VALUES FOR THE SIGNIFICANT INTERACTION IA \times INTERNODE

Relation to treated leaf	No. internode from base	IA γ per plant			Total
		0.0*	100	1000	
Below	1	22	30	41**	93
	2	27	30	23	80
	3	37	30	18**	85
	4	29	25	7**	61
Above	5	21	10	0**	31
	6	6	0	0	6
Totals		142	125	89	
L.S.D. 5%		16 for totals of 21 items 33 for totals of 126 items			30

* Values in this column are for 2,4-D alone.

** Mixtures of IA and 2,4-D significantly different from those shown for 2,4-D (third column).

between the cotyledons and the first leaf. Data in Table VI are based on treatments involving 100 to 10007 IA and 1 to 107 2,4-D. Dosage-response curves for the mixtures containing 1007 IA and 1 to 107 2,4-D appear in Figure 1.

Leaf applications of 0.001 ml. Plants treated with 0.001 ml. of solutions containing 0.1 to 17 2,4-D induced responses of a slightly higher magnitude than those resulting from a 0.01 ml. application containing the same quantity of 2,4-D. This difference is presumably due to the higher concentration of 2,4-D (ten times) used with the 0.001 ml. application without involving a corresponding calculated decrease of ten times in the size of the treated area (5). Results in Figure 4 show that the minimum effective dose of 2,4-D for increase in growth and modification lies between 0.01 and 0.17 and for the induction of proliferation between 0.1 and 17.

The minimum dose of IA which inhibited the action of 2,4-D lies be-

tween 10 and 100 γ . A mixture containing 100 γ IA and 1 γ 2,4-D caused approximately a ten-fold reduction in growth, modification, and proliferation. This represented nearly complete counteraction of the effect of 2,4-D. The appearance of these plants 14 days after treatment is illustrated in Figure 5. Responses on the plant treated with a mixture of 100 γ IA and 1 γ 2,4-D (lower right) are essentially the same as on the plant treated with 0.1 γ 2,4-D (upper left).

Changes in stem curvature up to 96 hours after treatment are shown

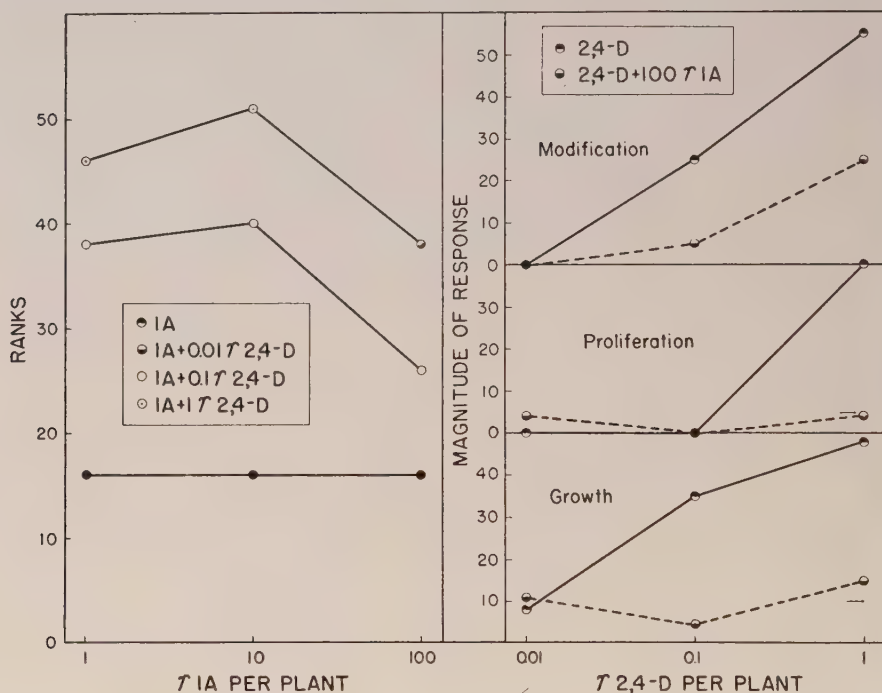


FIGURE 4. Influence of dosage level of IA and 2,4-D in causing additive and inhibitive effects on tomato plants treated with 0.001 ml. of the test solution. Responses evaluated 15 days after treatment.

for 0.001 ml. applications of IA and 2,4-D in Figure 6. Initial curvatures induced by mixtures of IA and 2,4-D showed additive effects at the lowest dosage level of 2,4-D and inhibitive effects at the two higher dosage levels. At the end of 2 hours, 1 γ 2,4-D caused no curvature yet the same quantity of 2,4-D in the mixture prevented IA from inducing stem curvature. After 3.5 hours the same plants exhibited a ten-fold reduction in curvature, and they continued to show less curvature up to 96 hours, as compared with plants treated with an equivalent dose of 2,4-D.

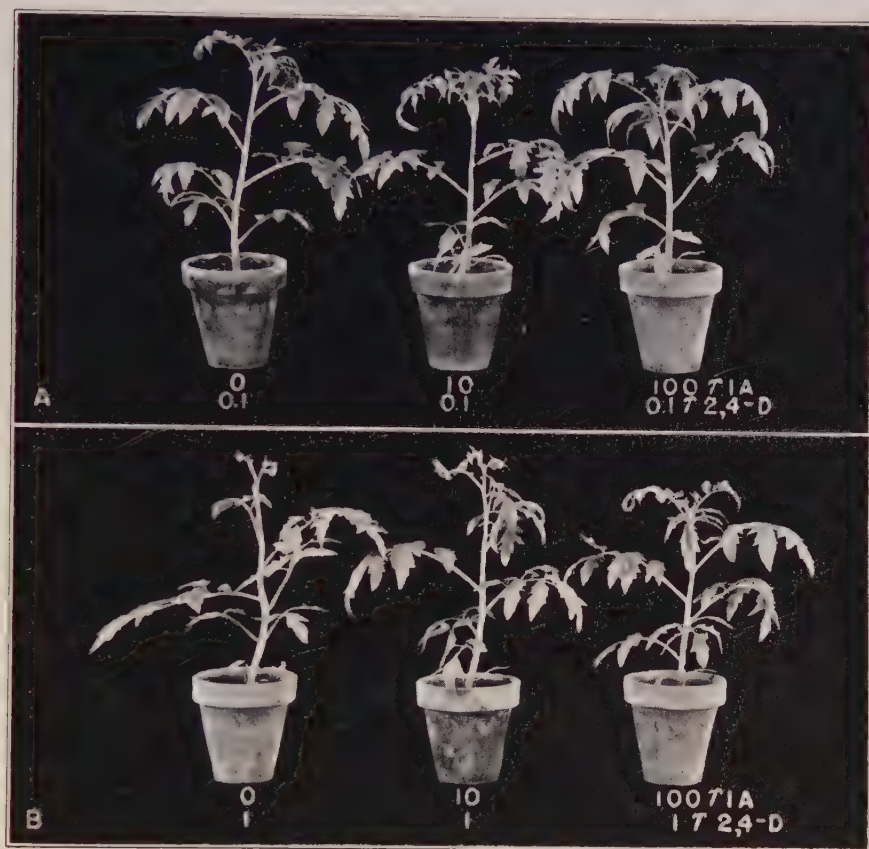


FIGURE 5. Response of tomato plants, after 14 days, to a 0.001 ml. application of solutions containing from left to right, A. 0.17 2,4-D, 0.17 2,4-D+100 7IA, and 0.17 2,4-D+100 7IA. B. Same as in A, but with 17 2,4-D.

In the test just described the greatest additive effects on stem curvature, measured up to 48 hours after treatment, were obtained with mixtures containing 0.01 to 0.17 2,4-D and 10 to 100 7IA (not illustrated in Fig. 6). For example, after 3 hours plants treated with a mixture of 0.17 2,4-D and 100 7IA exhibited an average curvature of 47° as compared to 25° on plants treated with 100 7IA and 3° for plants treated with 0.17 2,4-D. The plants treated with this mixture of 100 7IA and 0.17 2,4-D also exhibited additive effects at the end of 14 days (Fig. 4). This is in contrast to plants treated with a mixture of 100 7IA and 17 2,4-D which showed a lesser degree of stem curvature and also a lower magnitude of responses after 14 days (Fig. 4). These results are in agreement with those previously

described for 0.01 ml. applications in showing that additive effects are generally obtained with the lower doses of 2,4-D and inhibitive effects with higher doses.

In one series of tests tomato plants were treated with 0.01 to 3.27 2,4-D before or after being placed in a horizontal position for 1 and 3 days respectively and then returned to the upright position. The response

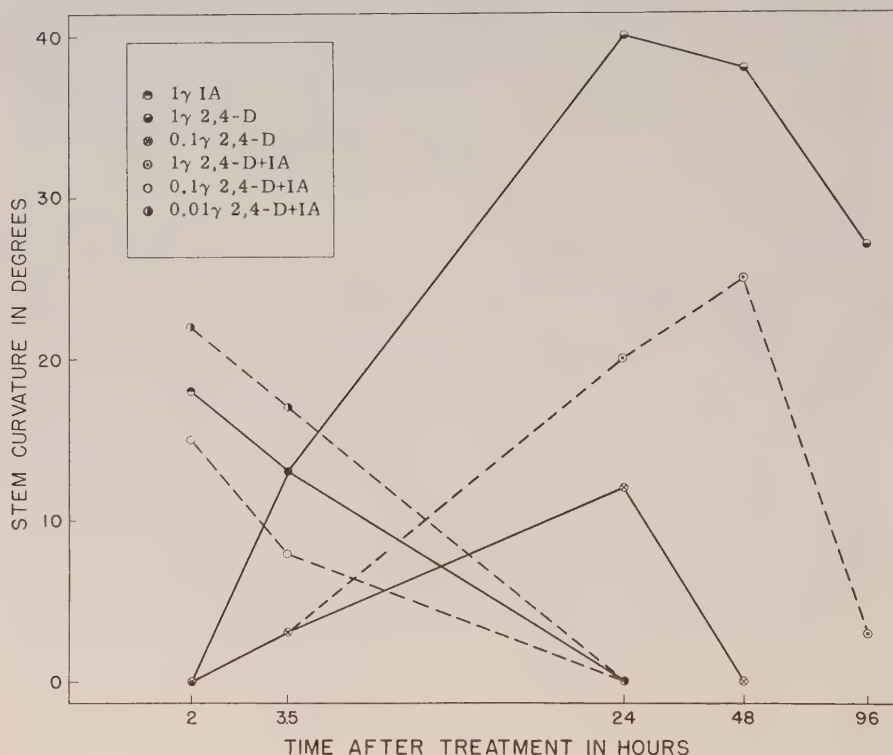


FIGURE 6. Changes in stem curvature during a 4-day period following treatment of tomato plants with a 0.001 ml. application of IA, 2,4-D, and mixtures of IA and 2,4-D.

to gravity and responses evaluated 8 to 17 days later showed that the treatment with 2,4-D induced additive or inhibitive effects, depending upon the dose of 2,4-D, the duration of the gravity stimulus, and the position of the treated leaf while the plants were in the horizontal position. Placing the treated leaf on the lower side hastened the upward curvature of stems, whereas placing the treated leaf on the upper side retarded or prevented the plant from responding to gravity. Later, after the plants had been restored to their upright position, it appeared that different responses (proliferation and modification) were affected independently in some cases and the same responses (growth, curvature, and proliferation) in other

cases. Thus the consistent relation between growth, stem curvature, proliferation, and modification responses on upright treated plants did not hold for similarly treated plants subjected to the gravity stimulus. In some respects these results appear to be similar to those obtained with the combined action of virus and 2,4-D in bean and aster plants (2). However, with respect to curvature and proliferation of stems, the present results are similar to those described for plants treated with IA, NA, and indole-

TABLE VII

COMPARATIVE RESULTS OBTAINED WITH MICROPIPETTES AND PLATINUM LOOPS USED TO APPLY 2,4-D TO ONE LEAFLET ON A TOMATO PLANT

Amount of 2,4-D applied per plant		Stem curvature after 48 hr., degrees		Magnitude of responses after 14 days					
				Height increase, mm.		Relative amount			
						Leaf modification		Proliferation	
γ	ML.	Pipette	Loop	Pipette	Loop	Pipette	Loop	Pipette	Loop
0	0.001	0	0	208	217	0	0	0	0
	0.01	0	0	225	210	0	0	0	0
0.1	0.001	0	0	235	264	0	0	0	0
	0.01	0	0	259	245	5	0	0	0
0.32	0.001	8	0	260	257	15	15	0	0
	0.01	0	0	230	237	15	10	0	0
1.0	0.001	12	20	255	243	35	40	0	0
	0.01	15	10	247	240	30	30	0	0
3.2	0.001	20	23	248	247	50	45	0	0
	0.01	23	20	253	270	50	50	5	5
10	0.001	67	65	209	249	40	50	25	25
	0.01	70	52	165	253	40	55	35	20
32	0.001	35	35	63	73	0	0	55	55
	0.01	47	50	65	68	0	0	55	55
100	0.001	0	20	55	64	0	0	60	60
	0.01	0	27	52	62	0	0	60	60

butyric acid (IB) before or after being subjected to a light or gravity stimulus (10, 11).

Comparative results involving the application of 0.001 and 0.01 ml. 2,4-D by means of a pipette and a loop are shown in Table VII. Responses evaluated after 14 days were essentially the same for each method of application on the basis of the γ 2,4-D applied per plant. The slight differences obtained at certain dosage levels are probably due to the difference in action resulting from the deposition of a droplet by means of a loop as compared with a pipette. Test solutions were applied more quickly and with greater ease by means of a loop than with a pipette. Treatment of 66 plants

by means of a loop, involving 22 different test solutions, required only 15 minutes. This is the only case in which both the pipette and loop were used to apply volumes of 0.001 and 0.01 ml. respectively, in the same test. In view of the satisfactory results obtained with platinum loops, it is planned to use them instead of pipettes in future tests.

Combination soil and leaflet treatments. Various combinations of leaf and soil applications of IA and 2,4-D resulted in the same magnitude of responses induced by either substance used alone or the effects were additive as in the case of applying IA and 2,4-D to separate areas on the foliage. This is in contrast to the inhibitive and additive effects obtained by adding 1-NOA to the soil and 2,4-D to a leaflet. In these latter tests 1 and 10 mg. 1-NOA respectively were added to the soil in combination with leaflet applications of 17 and 107 2,4-D. With a leaflet application of 107 2,4-D, a soil application of 1 mg. 1-NOA increased and a 10 mg. dose decreased the degree of stem curvature. Proliferation was decreased only by the 10 mg. dose of 1-NOA. Apparently all substances do not function the same when added to the soil in combination treatments involving application of 2,4-D to a leaflet.

When mixtures of IA and 2,4-D were added to the soil in lethal or near lethal doses, the effects were additive as indicated by a faster rate of killing or by killing when an equivalent dose of either 2,4-D or IA did not. Other compounds besides IA also give similar results. These particular results are similar to those obtained with spray solutions (4).

Other combination treatments. When used in combination with 2,4-D, the following compounds functioned as activators and inhibitors: 1-NA, 2-NA, 1-NOA, 2-NOA, α -(2,4-dichlorophenoxy)propionic acid, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,3,5-triiodobenzoic acid (TIB), maleic hydrazide, sodium 2,4-dichlorophenoxyethyl sulfate, cinnamic acid, sodium fluoride, iodoacetic acid, and monochloroacetic acid. Additive and inhibitive effects were obtained when the following compounds were used in combination with 1-NOA: IA, 2,4-D, α -(2,4-dichlorophenoxy)propionic acid, 2,4,5-T, 1-NA, 2,3,5-triiodoacetic acid, maleic hydrazide, and 2-NOA. The most pronounced inhibitive effects were obtained when a mixture of the two substances was applied to one or two leaflets as in the case of mixtures of IA and 2,4-D. The mixture which generally induced the most pronounced inhibitive effects consisted of 100 to 3207 of the inhibitor and 107 of the growth regulator being inhibited.

Herbicidal effects. Crop plants used in the pre-emergence flat tests were as follows: buckwheat (*Fagopyrum esculentum* Moench.), oat (*Avena sativa* L.), radish (*Raphanus sativus* L.), alfalfa (*Medicago sativa* L.), and regular lawn grass mixture of O. M. Scott & Sons Co. The following species of weeds emerged in control flats: purslane (*Portulaca oleracea* L.), chick-week (*Stellaria media* [L.] Cyrill), *Oxalis* sp., galinsoga (*Galinsoga parvi-*

flora Cav.), smartweed (*Polygonum pennsylvanicum* L.), crab grass (*Digitaria* sp.), annual bluegrass (*Poa annua* L.), and unknown species of grasses.

When 1 per cent 1-NOA was added to the soil of potted tomato plants, the surface remained free from broad-leaved weeds for two weeks or more, whereas the weeds originally present in control soil continued to grow and new weeds emerged. Similar results were obtained when the surface of soil in flats was sprayed with 1-NOA at the rate of 16 to 22 ml. per flat (approximately 2 lb. per acre). After 21 days the control flat contained 141 broad-leaved weeds and 254 grass seedlings. The treated flats contained 7, 15, and 2 broad-leaved weeds and 148, 157, and 82 grass seedlings in flats treated respectively with 0.32, 1, and 3.2 per cent 1-NOA. This represented a control of 90 to 98 per cent of the broad-leaved weeds but only 43 to 68 per cent of the grasses. Another series of flats was sprayed at the same time immediately after seeding to buckwheat, oats, radish, alfalfa, and Scott's lawn seed. There was no noticeable adverse effect on germination and growth of these crops during the first three weeks. Thereafter, the grass, alfalfa, and radish appeared to be slightly retarded but still retained their normal green color. Oat and buckwheat plants were not retarded.

DISCUSSION

The responses induced by 2,4-D exhibited a close correlation over a relatively wide range of dosage (Fig. 1 and 4, and Tables I and IV). Thus, with increasing doses of 2,4-D, growth first increased, then was retarded, and finally inhibited. Leaf modification was the most sensitive response, being induced by as little as 0.17 2,4-D per plant (Fig. 4). Since modification of leaves depended upon growth, the magnitude of modification followed growth rather closely. However, modification was induced by lower doses of 2,4-D than were required to induce a significant increase in growth. Doses of 2,4-D which inhibited growth also delayed or prevented the development of modified leaves. In contrast, increasing the dose of 2,4-D throughout the non-lethal range continued to increase the magnitude of proliferation. These results are in agreement with those previously reported (5) in showing that the relationships between the several responses induced on tomato plants by 2,4-D are relatively the same, even though the threshold value for a given response varies according to the conditions under which the test plants are grown and the method of applying 2,4-D.

Since treatment of tomato plants with IA in combination with 2,4-D resulted in additive and inhibitive effects corresponding to responses induced by higher or lower doses of 2,4-D, as compared with the quantity in the mixture (Fig. 1), it follows that IA and 2,4-D do not function alike when applied as growth regulators. Both compounds have the capacity to regulate the rate of cell elongation resulting in straight growth or curva-

tures, and to induce proliferation of tissue, but 2,4-D has, in addition, the capacity to modify leaves and other organs, whereas IA and naturally-occurring auxins do not possess this property. In the present tests IA appeared to function as a general stimulant in low doses and as a toxicant type of inhibitor in high doses without exerting a preferential effect on any of the responses in question. An additional difference between IA and 2,4-D related to their effect on stem elongation in which case an optimal dose of IA caused only a slight increase (10 to 20 mm.) over that of controls, whereas 2,4-D caused substantial increases of 60 mm. or more (Fig. 1).

Responses induced by IA and 2,4-D were essentially the same as those induced by mixtures containing 2,4-D and one of the following: 1-NOA, cinnamic acid, sodium fluoride, or iodoacetic acid. Since 1-NOA also inhibited the action of 2-NOA, IA, 2,4,5-T, TIB, 1-NA, α -(2,4-dichlorophenoxy)propionic acid, and maleic hydrazide, it is obvious that the inhibitive effects obtained in these tests do not depend upon structural specificity.

Additive effects not involving an increase in leaf modification were previously obtained with non-lethal spray solutions containing 0.0001 per cent 2,4-D and either 0.032 per cent diallyl maleate or 0.32 per cent ammonium thiocyanate (4). Inhibitive effects were not obtained with higher doses of these mixtures. Apparently combination treatments with spray solutions do not always give results comparable to those obtained with leaflet applications. However, in other tests (12) 5-chloro-1,2,3,2*H*-benzotriazole-2-acetic acid inhibited the action of 2,4-D when the two compounds were applied in mixtures or separately as successive spray treatments.

Changes in the degree of modification and the relative amount of proliferation on 2,4-D-treated plants subjected to the gravity stimulus indicated that the combined action of naturally-occurring auxins and applied 2,4-D was not the same as on similarly treated upright plants. Since proliferation was affected independently of leaf modification on plants subjected to the gravity stimulus, it would appear that naturally-occurring auxins produced or redistributed under the influence of gravity have no direct effect on the mechanism responsible for modification. Thus the effect of 2,4-D on curvature and proliferation responses on plants subjected to the gravity stimulus was similar to that of IA, NA, and IB (10, 11) which are active for curvature and proliferation but are not active for modification.

In the case of combination treatments applied to upright plants, changes in modification can be explained on the basis of the relative effectiveness of non-specific substances such as IA, NA, 1-NOA, cinnamic acid, iodoacetic acid, and sodium fluoride in affecting the mechanism which

inactivates 2,4-D without directly affecting the mechanism causing modification. This explanation precludes the possibility that applied IA or naturally-occurring auxins of the IA type have any direct effect on the leaf-modifying mechanism. The close correlation between modification and proliferation resulting from combination treatments with IA and 2,4-D (Fig. 1, 2, and 4) is in support of this concept, since the results are accounted for on the basis of the relative amount of 2,4-D inactivated. Repression of the inactivating mechanism would lead to additive effects and activation of the mechanism would lead to inhibitive effects.

Results of tests in which the treated leaf was removed showed, as in the case of intact plants, that IA did not act preferentially to change the magnitude of growth or proliferation without affecting the magnitude of modification. Particularly in the cases of high dosage levels of 100 γ or more of 2,4-D, removal of the leaf 1 to 15 minutes after treatment resulted in more pronounced responses than when the treated leaflet was removed. Presumably the non-treated portion of the treated leaf plays some part in counteracting the action of 2,4-D. Possible interference with the translocation of 2,4-D from the treated leaf into the stem is not precluded.

Although small quantities of 2,4-D were applied to each plant, the concentrations were relatively high, being in some cases equivalent to those used in herbicidal sprays. For example, 0.001 ml. of a 0.1 per cent solution contained 1 γ 2,4-D. A lethal dose of spray solution for tomato seedlings (3 ml. of 0.1 per cent 2,4-D) contained 3000 γ 2,4-D (5). However, as is shown by data in Table I, it is not necessary to use lethal doses when comparing different formulations of 2,4-D. Furthermore, differences in stem curvature occurring 24 to 48 hours after treatment can generally be used as an approximate measure of the relative effectiveness of different formulations since the evaluations based on stem curvature (Fig. 6) were relatively the same as those based on all responses exhibited one to two weeks later (Fig. 4).

SUMMARY

Responses induced on tomato plants by applying IA in combination with 2,4-D varied according to the morphological position of the treated leaflets and the quantity of 2,4-D and IA applied. Additive effects generally resulted from the use of 1 γ 2,4-D or less and inhibitive effects with higher doses of 2,4-D. Increasing the distance between the areas treated with IA and 2,4-D either decreased the magnitude of inhibition or caused additive effects, and, conversely, the closer the treated areas were, the greater the inhibitive effects.

Depending upon whether IA functioned as an activator or inhibitor, the responses induced by mixtures of IA and 2,4-D were qualitatively and quantitatively the same as the responses induced by a higher or lower dose

of 2,4-D. Since IA does not modify leaves, any reduction in the magnitude of modification and other responses induced by mixtures containing more than 17 2,4-D appeared to involve inactivation of 2,4-D.

Results obtained with IA and 2,4-D were essentially the same as those induced by mixtures of 2,4-D and either 1-NOA, cinnamic acid, sodium fluoride, or iodoacetic acid. 1-NOA also inhibited the action of 2-NOA, IA, 2,4,5-T, TIB, 1-NA, α -(2,4-dichlorophenoxy)propionic acid, and maleic hydrazide. Thus the activation or inhibition of the action of 2,4-D or other growth regulators was of a general nature which did not depend upon structural specificity of the activator or inhibitor.

Treatment of the soil with mixtures of IA and 2,4-D, or adding one substance to the soil and the other to one leaflet, resulted in additive effects under certain conditions but not inhibitive effects.

The response of tomato plants to gravity and the responses evaluated one to two weeks later showed that the additive and inhibitive effects induced by 2,4-D depended upon the dose of 2,4-D, the duration of the gravity stimulus before or after treatment, and the position of the treated leaf while the plants were in the horizontal position. Different responses (modification and proliferation) were affected independently in some cases and the same responses in other cases, showing that the consistent relation between the responses on upright treated plants did not hold for similarly treated plants subjected to the gravity stimulus.

Test solutions were applied faster and with greater ease by means of fused platinum true circle loops delivering 0.001 and 0.01 ml. respectively, than by micropipettes.

1-NOA was an effective pre-emergence herbicide in greenhouse tests when applied at the rate of about 2 lb. per acre as a pre-emergence treatment, giving 90 to 98 per cent control of broad-leaved weeds for at least three weeks.

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AN OBJECTIVE METHOD FOR INSECTICIDE BIOASSAY BASED ON PHOTOMIGRATION OF MOSQUITO LARVAE¹

H. P. BURCHFIELD, J. D. HILCHEY, AND ELEANOR E. STORRS²

Since the publication by Nolan and Wilcoxon (8) of a method for the bioassay of parathion using larvae of *Aedes aegypti* (L.), widespread interest has been aroused in this sensitive and versatile technique both from the standpoints of detecting toxic spray residues in foods and evaluating new insecticidal chemicals. Hartzell and Storrs (5) adapted the method to the detection of a wide variety of organic insecticides both in pure suspension and in the presence of food extracts and found it to be generally useful. Subsequently, work was undertaken to study causes of variability in test results and to devise new techniques for improving precision. Several sources of error are inherent in the test procedure. The number of larvae at each dose is frequently too small to provide a representative sampling of the parent population, and when large groups are used the estimation of mortality becomes burdensome. Furthermore, different observers may not always agree on when death occurs since there are often many moribund individuals difficult to classify.

To provide an objective method suitable for handling large populations, a number of physiological responses of larvae were investigated. A stimulus was desired that could be used to segregate test organisms according to degree of moribundity without the exercise of personal judgment on the part of the operator. A rapid procedure based on the tendency of mosquito larvae to move away from a strong light source was finally developed.

This paper briefly reviews some of the difficulties inherent in estimating mortality by visual observation, and describes the equipment and techniques used to assess inhibitory effects objectively through photomigration. Preliminary results on the recovery of insecticidal residues from raw and processed foods are presented, and factors influencing the accuracy of test results discussed in detail. A news report on this method has been published elsewhere (1).

MATERIALS AND METHODS

REARING LARVAE OF *Aedes aegypti* (L.)

The early part of this work was carried out with dry eggs shipped from the United States Department of Agriculture station at Orlando, Florida.

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Prior to use they were conditioned by sprinkling them in thin layers on moistened circlelets of filter paper which were then stored in a closed container. Eggs treated in this way hatched satisfactorily at the end of three days, and in most cases were still viable at the end of a month. Later, an adult colony was established from the Orlando strain to provide a constant source of eggs which had not undergone a dryingout process. The culture was maintained by the usual methods (4).

Mature eggs on filter paper circlelets were hatched in 25 ml. of culture medium from a previous rearing. After 20 minutes the paper and unhatched eggs were removed, and the larvae counted out into groups of 250.

A culture medium was prepared by mixing 0.5 g. Fleischmann's dry baking yeast, 0.15 g. blood albumin (Fisher c.p.) and 0.10 g. sucrose into a thin paste with a little water. The paste was diluted to 1000 ml. with water and placed in a 12"×7"×2" enameled photographic tray. Two hundred and fifty larvae were added to each of a series of trays, and incubated for 44 hours at $29^{\circ} \pm 0.5^{\circ}$ C.

On removal from the cabinet the trays were illuminated unevenly so the larvae congregated in the shaded areas. They were taken up with a medicine dropper and pooled, and then transferred to a 60-mesh screen and washed with demineralized water to free them from nutrient. Groups of 200 larvae were counted out and stored in the dark at room temperature in *ca* 25 ml. of water until used. Larvae treated in this way maintained constant resistance over a normal working day.

Careful attention was given to temperature control, cleanliness, scheduling, and minor details in making up nutrients to minimize day-to-day fluctuations in resistance to toxicants.

ESTIMATION OF MORTALITY BY VISUAL OBSERVATION

In order to evaluate the errors inherent in assessing mortality by visual observation, experiments were carried out with pure suspensions of insecticides using a modification of the technique described by Hartzell and Storrs (5). Acetone solutions of parathion, methoxychlor, DDT, and aldrin were diluted with water in concentration series established by preliminary experiments. Fifteen ml. aliquots were placed in test tubes and *ca* ten larvae added to each tube. After 24 hours' incubation at 29° C. the contents of the tubes were transferred individually to porcelain dishes and the mortality judged by three independent observers. Larvae which moved spontaneously were counted as alive, while those which were sluggish and responded only when disturbed or touched with a probe were counted as moribund. One of the observers classified the larvae as dead or alive only. Data obtained in replicated experiments are discussed in the experimental section.

PHOTOMIGRATION TECHNIQUE FOR MEASURING TOXIC ACTION

Toxic action was measured by confining mosquito larvae behind a porous barrier one inch from the end of a transparent trough containing an aqueous suspension of the test chemical. A light source adjacent to the blockedoff compartment was turned on and the barrier removed. Unaffected larvae swam away from the light to the far end of the trough, while those that were dead or moribund failed to migrate or migrated slowly. At the end of one minute a second barrier was dropped into place three inches from the end of the trough nearest the light, and the larvae confined behind were regarded as inactivated by the toxicant.

Equipment. The source of light for photomigration was a 500-watt

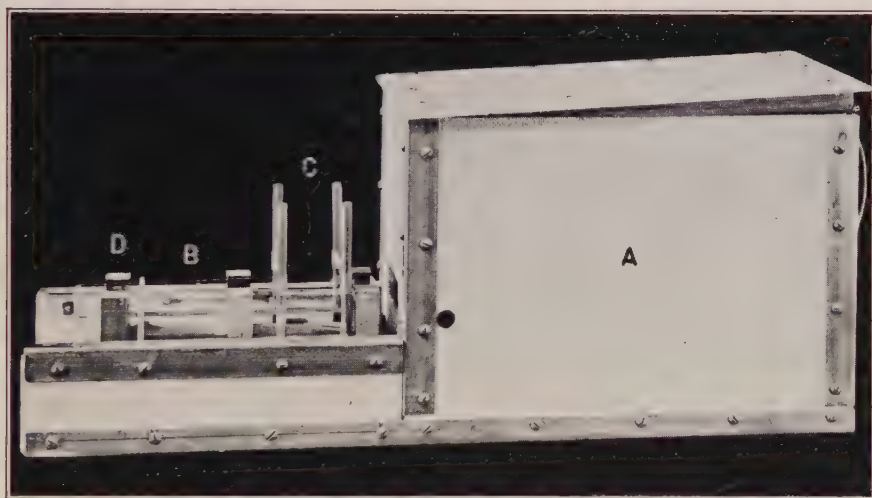


FIGURE 1. Apparatus for measuring response of mosquito larvae to light. (A) Lamp housing. (B) Migration chamber. (C) Barrier posts. (D) Chamber support.

General Electric projection lamp housed in a Flexboard box 14" long, 10½" high, and 8½" wide. A shelf 8½" wide, 11" long, and 3⅜" high was attached to the front of the box to support the migration chamber. An opening to accommodate the narrow end of the chamber was cut into the box on the level of the shelf. The lamp was placed so the center of the filament was on the same plane as the end of the chamber. The distance from the near surface of the bulb to the surface of the chamber was six inches. A circulating water filter was placed inside the box just behind the opening to prevent warming of the test solution.

Preliminary experiments on the migration of larvae in the absence of toxicants were carried out in a Lucite tray constructed like a microscope slide box, with the sides grooved at one-inch intervals to accommodate

barriers. However, this equipment was not suitable for bioassay since insecticides are absorbed by the plastic. To avoid this, glass migration chambers were made by sealing off a ten-inch section of 50 mm. Pyrex tubing with glass windows and then cutting it longitudinally to form two semi-circular troughs. The troughs were mounted in wooden cradles equipped with pairs of $\frac{1}{4}'' \times 5''$ brass uprights spaced one and three inches from the end near the light source to serve as guides for the barriers (Fig. 1).

The barriers were cut from 0.05 inch stainless steel plate. They were equipped with horizontal arms terminating in rings to slide along the upright posts so that they could be dropped into position to partition off the trough. The lower section of each barrier was cut into the form of a semi-circle to make a snug fit with the trough, and then perforated so that the solution on each side would reach a common level when making transfers.

Materials tested. During the course of this work toxicity data were obtained on DDT [2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane], methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane], lindane (γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane), aldrin [1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene], chlordan [1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene and impurities], heptachlor [1 (or 3a), 4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene], parathion [*O,O*-diethyl-*O-p*-nitrophenylthiophosphate], pyrethrins, nicotine sulfate, and sodium cyanide. The chlorinated hydrocarbons, parathion and pyrethrins were tested in the range of 0.02 to 1.00 p.p.m., and nicotine and sodium cyanide at 10 to 100 p.p.m.

The organic insecticides were made up in acetone solution at 1000 p.p.m. and serially diluted down to 1 p.p.m. with acetone at concentration ratios determined by the toxic properties of the individual compounds.

Measurement of inactivation rate. One ml. of an acetone solution containing insecticide was added to 49 ml. of water and mixed thoroughly. The diluted insecticide was then added to 50 ml. of water containing 200 larvae of *Aedes aegypti* and the time of mixing recorded. The suspension was then transferred to the migration chamber and the light turned on. Any larvae which failed to migrate were removed and discarded at this time. The remaining larvae were tested from time to time by flashing on the light and when the first evidences of inactivation occurred, a barrier was placed in position at the one-inch mark and the light turned off. All of the larvae were then transferred with a medicine dropper or pipette to the space between the barrier and the end of the trough adjacent to the light source. The barrier was removed three seconds before the nearest minute on the second hand of a clock. The light was turned on three seconds later and the time recorded. After exactly one minute a second barrier was dropped into place at the three-inch mark and the light turned off. If more than *ca* 20 larvae were entrapped they were removed from the chamber

with a medicine dropper and counted. Otherwise they were retained for the next reading. The entrapped larvae were filtered out on a plug of glass wool placed in the stem of a 10 cm. funnel and the suspension returned to the trough. The barrier was replaced at the one-inch mark and the surviving larvae transferred to the space between it and the end of the chamber. This procedure was repeated at intervals until 80 to 90 per cent of the larvae were inactivated. Total elapsed time and cumulative percentage of moribund larvae were recorded at each reading.

Serial dilution method. A series of dilutions of an aqueous suspension of insecticide was prepared and larvae added to 50 ml. portions at each concentration. They were made up to 100 ml. and held in an incubation chamber at 29° C. for 24 hours. At the end of this period each suspension was transferred to a migration chamber and the larvae confined in the space between the one-inch screen and the end of the chamber. When turbulence subsided, the screen was removed and the light turned on. At the end of exactly one minute, a barrier was interposed at the three-inch mark. The inactivated larvae were removed with a pipette and filtered through a Buchner funnel onto cross-hatched paper. The rate of filtration was regulated so the larvae were distributed uniformly on the paper. They were counted with the aid of a hand tally counter and the percentage inactivated at each concentration computed.

EXTRACTION OF INSECTICIDE RESIDUES FROM FOODS

In order to extract processed foods containing insecticide residues with toxicity equivalent to 0.5 p.p.m. DDT, 100 g. samples were diluted with 100 ml. of water and mixed with 200 ml. of *n*-hexane for two minutes in a Waring blender. The emulsions were then centrifuged in 500 ml. tubes for one-half hour at 1500 r.p.m. The clear supernatants were pipetted off and stored at 10° C. in glass stoppered bottles until used. Surface residues were stripped from leaves by placing 100 g. samples in 2-liter glass stoppered bottles containing 400 ml. *n*-hexane and inverting and shaking 50 times. The hexane was then decanted and reserved for purification and testing.

Plant pigments including degraded chlorophylls, carotenoids and xanthophylls were removed by adding 40 ml. of extract (equivalent to 20 g. of processed food) to 3×10 cm. chromatographic columns packed with calcium carbonate (U.S.P. precipitated Mallinckrodt). After the solvent percolated through, the columns were washed with 75 ml. of hexane and the percolates collected. The plant pigments were retained on the columns while part of the insecticide, together with lipids, were found in the percolates. The hexane was then evaporated in a current of air at room temperature taking care not to let the residues go completely to dryness or stand in air. In some experiments solid fats and waxes were partially removed by redissolving the residues in 5 ml. of acetone and freezing them

out in a salt-ice bath. One-half ml. Carbitol was added to the supernatants and the acetone evaporated under a current of air. The residues were re-dissolved in 1 ml. portions of acetone and diluted to 50 ml. by the addition of water. They were then added to 50 ml. of water containing 200 larvae of *Aedes aegypti* and inactivation rates determined as previously described. Recovery of insecticide was not quantitative and toxic action was partly masked if lipids were present in the final test solution.

EXPERIMENTAL RESULTS AND DISCUSSION

PRECISION OF MORTALITY COUNTS BY VISUAL OBSERVATION

Before proceeding with the development of a method to measure the

TABLE I

MORTALITY OF MOSQUITO LARVAE AFTER 24-HOUR EXPOSURE TO FOUR INSECTICIDES IN SERIAL DILUTION. AVERAGE RESULTS ON REPLICATES RECORDED BY THREE OBSERVERS

Concn. (p.p.m.)	Observer A				Observer B				Observer C			Av.
	% Mor- tality*	s	s _x	% Mori- bund	% Mor- tality	s	s _x	% Mori- bund	% Mor- tality	s	s _x	
Parathion												
0.0156	100.0	—	—	0	100.0	—	—	—	100.0	—	—	100.0
0.0078	99.0	—	—	2	100.0	—	—	—	99.5	—	—	99.5
0.0039	71	17.8	3.6	15	73	15.0	3.0	8	68	21.8	4.4	71
0.0019	25	14.0	3.1	9	26	13.8	3.1	6	25	18.4	4.1	25
0.00095	2	—	—	7	3	—	—	4	1	—	—	2
0.00047	1	—	—	1	2	—	—	2	0	—	—	1
Methoxychlor												
0.250	99.0	—	—	—	100.0	—	—	—	99.0	—	—	99.3
0.125	97.0	—	—	—	99.0	—	—	—	100.0	—	—	98.7
0.0625	85	—	—	26	95.0	—	—	4	99.0	—	—	93.0
0.0313	62	10	2.3	33	66	13	3.0	19	56	18	4.1	61
0.0156	36	18	4.0	31	33	19	4.3	18	27	18	4.0	33
0.0078	19	10	2.4	17	17	12	2.8	11	21	21	5	19
DDT												
0.025	98.6	—	—	4	99.2	—	—	4	100.0	—	—	99.3
0.0125	93.2	—	—	13	96.5	—	—	11	100.0	—	—	96.6
0.00625	74	13.8	3.1	48	83	14.5	3.2	40	91.0	9.4	2.1	84
0.003125	47	20.7	4.8	42	63	13.7	3.1	54	51	29.6	6.8	54
0.00156	18	—	—	20	24	—	—	27	9	—	—	17
0.00078	12	—	—	11	22	—	—	22	8	—	—	14
Aldrin												
0.0625	47	18.0	3.1	66	53	18.2	3.1	48	46	24.1	4.1	49
0.03125	53	8.5	1.5	80	61	11.2	1.9	44	55	25.7	4.4	56
0.0156	7	—	—	7	6	—	—	2	17	—	—	10
0.0078	7	—	—	1	11	—	—	3	21	—	—	13

* Averages of 20 replicates of 10 larvae each except for aldrin where 35 replicates were used.

effect of toxicants on mosquito larvae through response to light stimuli, the method of Nolan and Wilcoxon (8) and Hartzell and Storrs (5) was evaluated to determine its precision and dependence on subjective factors.

Tests were run in dilution series on parathion, methoxychlor, and DDT using ten groups of 20 larvae at each of five concentrations. Since results previously obtained on aldrin were erratic, this chemical was tested with 35 groups of ten larvae at each of four concentrations. Mortality was judged independently by three different observers to assess the import-

TABLE II
COMPARISON OF LD₅₀ VALUES AND SLOPES OF REGRESSION CURVES ON
THREE INSECTICIDES ASSAYED IN REPLICATE BY SERIAL DILUTION

Observer	LD ₅₀ (p.p.m.)	Slope
DDT		
A	0.00345	2.70
B	0.00250	2.22
C	0.00313	4.40
Average	0.00306	
Methoxychlor		
A	0.0230	2.36
B	0.0200	2.86
C	0.0270	2.39
Average	0.0233	
Parathion		
A	0.0029	4.10
B	0.0027	4.30
C	0.0029	3.91
Average	0.0028	

ance of personal differences in interpreting test results. Observers A and B classified the larvae as dead, alive, and moribund, while observer C classified them as dead and alive only. The term moribund was used to designate larvae that were visibly affected by the chemical but which were still capable of movement. Moribund larvae were arbitrarily weighted at 0.5 in the computation of mortality.

The average results obtained by the three observers (Table I) agreed satisfactorily for parathion but tended to diverge for the other insecticides. Thus the difference in average mortality recorded by B and C at 0.0313 p.p.m. methoxychlor was 10 per cent or almost three times the average standard deviation from the mean for the observers. At 0.00625 p.p.m. DDT the difference between A and C was 17 per cent or seven times the standard deviation. LD₅₀ values and the slopes of the dosage-re-

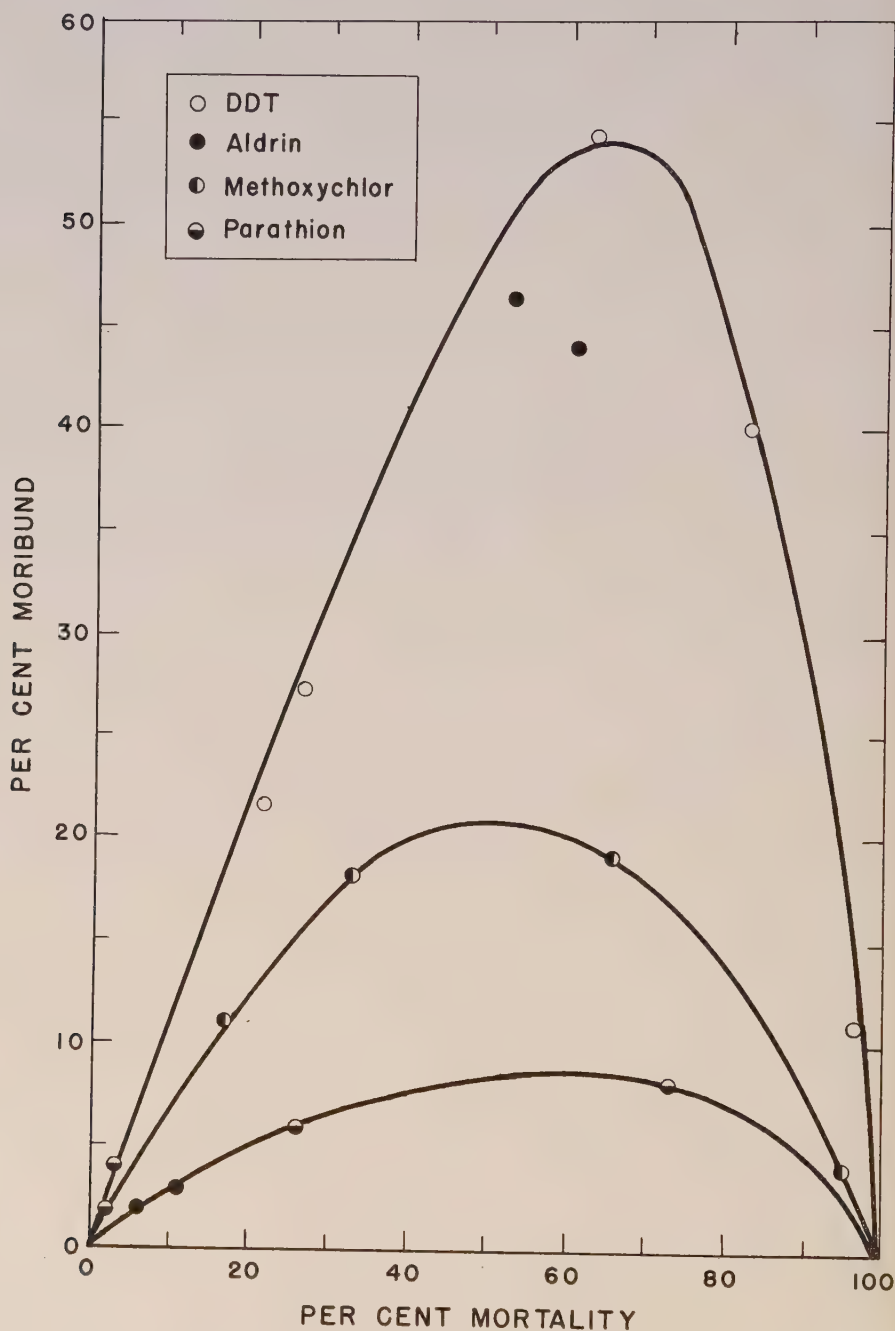


FIGURE 2. Variation in number of moribund larvae with respect to percentage mortality in serial dilution tests. Observer B.

sponse curves as plotted on logarithmic-probability paper (Table II) showed similar correspondence. Although the three observers agreed reasonably well on estimating the mortality caused by aldrin, the data in dilution series were irregular and could not be used for the computation of LD₅₀. This was in part caused by the fact that the rate of kill was slow, and many larvae which eventually would have died were recorded as moribund or alive although they were in a comatose condition.

Discrepancies between observers in judging identical groups of larvae were caused primarily by the presence of moribund individuals in regions

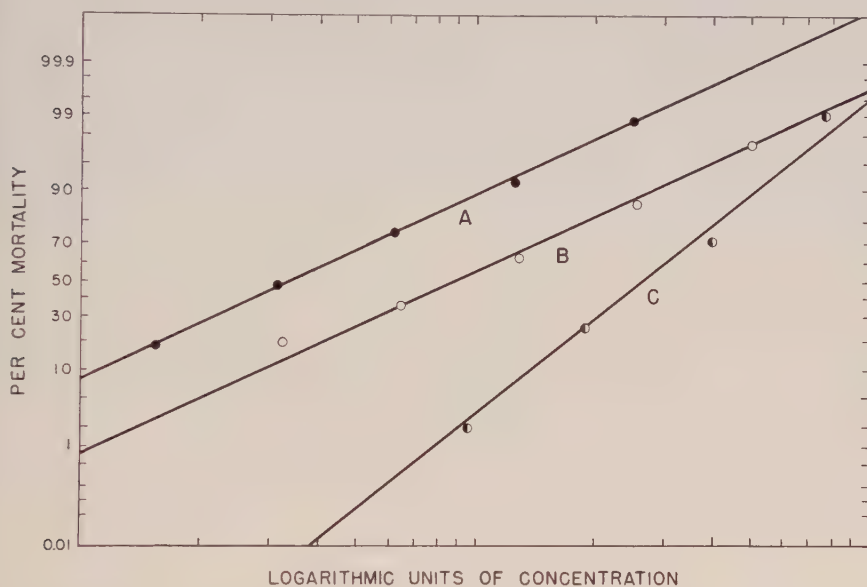


FIGURE 3. Dosage-response curves obtained by observer A using 200 larvae at each concentration of insecticide. Mortality estimated by visual inspection. (A) DDT. (B) Methoxychlor. (C) Parathion.

of intermediate kill. When the percentage of moribund larvae was plotted against percentage mortality, inverted U curves were obtained with maxima in the neighborhood of 50 to 70 per cent (Fig. 2). It is clear that the insecticides differ widely in their modes of action, and that these differences were reflected in the interpretation of test results. Thus moribundity increased in the order Parathion < Methoxychlor < DDT and agreement between observers decreased in the same order.

The dosage-response curves obtained on three of the four insecticides (Fig. 3) indicated that satisfactory results could be obtained if a sufficient number of replicates were used to minimize variations caused by non-representative sampling of the original larval population and differences in personal opinion in judging mortality.

However, tests carried out on this scale would be impractical for ordinary laboratory use. It is necessary, therefore, to examine the situation that would arise when smaller numbers of larvae are employed.

If larvae are withdrawn at random from a large population of known resistance, the standard deviation of sampling is

$$s_n = \sqrt{\frac{PQ}{n}} \quad (1)$$

where P is the proportion of the population that should be killed, Q the proportion that should survive, and n the number of larvae in the subsample.

The standard deviations between replicate tubes were computed for this experiment by the usual methods (Table I). In general, the values were higher than the sampling errors computed from equation (1) although there were occasional exceptions. Since the tubes were treated identically insofar as possible, the residual errors of estimation given by

$$s_r = \sqrt{s^2 - s_n^2} \quad (2)$$

must arise largely through differences in the personal judgment of the operators. Thus, throughout this experiment observers A and B were more precise than observer C in interpreting identical samples (Table III).

It can be concluded that the use of ten larvae at each dose is not enough for many applications since the standard deviation of sampling alone is about 14.5 per cent when the mortality is 70 per cent, and the total error is generally higher when observational differences are also included. Practical difficulties in segregation and counting make it inconvenient to use more than ten larvae per group, and when more than two or three groups are used at each concentration of insecticide, the test soon becomes unwieldy. It is evident, therefore, that a method by which large numbers of larvae could be segregated according to degree of moribundity through a standard physiological stimulus would improve precision and ease of manipulation. It would also eliminate personal differences between observers in evaluating test results and provide the basis for an objective method of bioassay.

These observations do not take into account variations in the day-to-day viability of the test larvae and erratic fluctuations in mortality sometimes incurred through accidental contamination of test solutions. These occurrences are frequently serious since the standard comparison sample and the unknowns must be made up at the same time and the results read after a 24-hour incubation period. If the optimum dosage range is missed through unexpected changes in the resistance of the larvae, or the results masked by the presence of impurities picked up from laboratory equip-

TABLE III
COMPARISON OF ERRORS INHERENT IN SAMPLING WITH ERRORS IN ASSESSING
RESULTS IN REPLICATE TESTS MADE ON FOUR INSECTICIDES WITH
THREE INDEPENDENT OBSERVERS

Observer	Concn. (p.p.m.)	% Mortality*	Standard deviation	Standard deviation of sampling	Residual error of estimation
Parathion					
A	0.0039	71	17.8	14.4	10.4
B		73	15.0	14.0	5.4
C		68	21.8	14.8	16.0
A	0.0019	25	14.0	13.7	3.0
B		26	13.8	13.8	0.0
C		25	18.4	13.7	12.3
Methoxychlor					
A	0.0313	62	10	15.4	<0
B		66	13	15.0	<0
C		56	18	15.7	8.8
A	0.0156	36	18	15.2	9.7
B		33	19	14.9	11.8
C		27	18	14.0	11.3
A	0.0078	19	10	12.4	<0
B		17	12	11.5	3.5
C		21	21	12.9	16.5
DDT					
A	0.0063	74	13.8	13.8	0.0
B		83	14.5	14.5	8.3
C		91	9.4	9.4	2.6
A	0.0031	47	20.7	15.8	13.4
B		63	13.7	15.2	<0
C		54	29.6	15.8	25.0
Aldrin					
A	0.0625	47	18.0	15.8	8.6
B		53	18.2	15.8	8.9
C		46	24.1	15.8	18.2
A	0.0313	53	8.5	15.8	<0
B		61	11.2	15.4	<0
C		55	25.7	15.7	20.4

* Averages of 20 replicates of 10 larvae each except for aldrin where 35 replicates were used.

ment, the entire experiment must be repeated. A test procedure that would allow for a rapid check on larval viability and be less sensitive to chance contaminants would have many obvious advantages.

PHOTOMIGRATION OF UNTREATED LARVAE WHEN EXPOSED TO LIGHT

It has long been known that mosquito larvae (2, p. 145) and other motile organisms in the lower phyla of the plant and animal kingdoms

(3, p. 423; 7) exhibit negative or positive phototaxis, but apparently this reaction has not been put to use in practical toxicological measurements.³

It seemed possible that if a toxicant were added to a solution containing free-swimming larvae, they would lose their ability to respond to light as neural and muscular paralysis progressed. The tendency to move away from light is enhanced if the source is unidirectional, for, from a point source, the intensity of light is inversely proportional to the square of the distance. Thus the larvae would be provided with a light density gradient along which they could move rather than having to reach a shaded area by chance. Furthermore, if the larvae were all started from an enclosed

TABLE IV
DISTANCES MIGRATED BY LARVAE AFTER EXPOSURE TO LIGHT
FOR DIFFERENT PERIODS OF TIME

Duration of exposure (minutes)	Number of larvae within distance (inches)					
	0-1	0-3	3-5	5-7	7-9	9-10
0.00	74	0	0	0	0	0
0.25	0	1	2	15	43	13
0.50	0	1	1	5	20	47
1.00	0	0	0	0	10	64
2.00	0	1	1	2	3	67
3.00	0	13	0	8	2	51
5.00	0	6	7	18	17	26

area near the light source, it should be possible to measure the percentage inactivated by counting the number remaining after releasing them. Before attempting to measure the action of insecticides, various factors affecting the photomigration of untreated larvae were investigated to set up arbitrary standards for the test.

A group of larvae in 100 ml. of water were placed in a 10"×3"×1" Lucite tray and enclosed behind a barrier at the one-inch mark. They were allowed to migrate for various periods after releasing them and turning on the light. At the end of each migration time, barriers were simultaneously introduced at one, three, five, seven, and nine inches and the larvae in each compartment counted.

The data (Table IV) showed that a migration time of one minute and a distance of three inches was satisfactory. When the time was less than one minute, some failed to cross the three-inch line, while after two minutes they tended to become adjusted to the light and moved back toward

³ After the completion of this work the authors learned that Dr. Ralph Heal of Merck & Co. has developed a similar procedure for the assay of lindane in technical benzenehexachloride. The data have not been published.

the starting point. The migration distance of three inches insures that almost all the members of a healthy population will cross the barrier line in the allotted time, and is great enough to prevent inactivated larvae from being carried across it by mechanical or convection currents.

The distribution of larvae within the chamber on repeated trials with

TABLE V
REPRODUCIBILITY OF CONSECUTIVE MIGRATION EXPERIMENTS
WITH THE SAME LARVAL POPULATION

Trial number	Number of larvae within distance (inches) after one minute				
	0-3	3-5	5-7	7-9	9-10
1	0	0	0	3	71
2	0	0	0	7	67
3	0	1	2	4	67
4	1	2	1	4	66
5	1	1	0	2	70
6	1	0	0	0	73

TABLE VI

EFFECT OF EXPOSURE OF LARVAE TO ALTERNATING PERIODS OF LIGHT AND DARK ON ABILITY TO MIGRATE IN CONSECUTIVE EXPERIMENTS WITH THE SAME POPULATION

Exposure before migration (min.)		Number of larvae within distance (inches) after one minute				
Light	Dark	0-3	3-5	5-7	7-9	9-10
0	0	1	0	0	0	73
1	0	15	4	8	11	36
0	5	0	0	5	4	65
1	0	3	9	8	15	39
0	5	0	0	1	13	60
2	0	16	19	12	7	20
5	0	9	6	6	10	43
0	120	9	5	8	15	37

the same population (Table V) was uniform. Thus larvae can be used in a series of tests before injury becomes a limiting factor. However, if they were confined close to the source and were illuminated for one minute before migration, response was much poorer since the intense light produced temporary desensitization (Table VI). If the exposures were short, recovery was practically complete after five minutes in the dark. However, on exposures up to five minutes, a two-hour rest period did not result in complete recovery. Thus, when it was necessary to have the light on for protracted intervals during counting and removal of inactivated larvae, an opaque barrier was used to protect the viable part of the population.

Although the best results were obtained when the lighting was unidirectional, diffuse sunlight or ordinary overhead illumination did not in-

terfere. However, when strong secondary illumination was applied, the larvae tended to move to a region where the net illumination was least. For instance, when a 500-watt source was placed opposite the normal source at a distance of two feet, the larvae congregated nearer the end of the chamber facing the more distant light.

The effect of wave length was not studied in detail although it was observed that migration decreased somewhat when a filter containing a solution of methylene blue was interposed between the source and the larvae. This may have been due as much to reduction in total intensity as to selective sensitivity. The effect of a water filter was also investigated since cutting down on the heat radiated from the lamp would tend to reduce temperature differences at the ends of the chamber, thus minimizing the possibility of larvae being carried by convection currents. Differences in

TABLE VII
EFFECT OF DEPTH OF WATER ON MIGRATION OF LARVAE

Volume (ml.)	Depth (inches)	Number of larvae within distance (inches) after one minute				
		0-3	3-5	5-7	7-9	9-10
25	0.051	44	52	41	28	42
50	0.101	0	1	5	50	152
100	0.202	0	2	2	6	197
200	0.404	0	0	0	4	203

the migration of untreated larvae were not significant. However, the use of a filter is recommended since it serves to prevent warming of the test solution during prolonged experiments.

The illumination provided by a 100-watt incandescent bulb was not intense enough for satisfactory migration. Most of the larvae moved away from the source, but the rate was slow and too many individuals failed to respond for adequate segregation. The 500-watt G. E. projection lamp proved to be entirely satisfactory and was used in all toxicity measurements.

The depth of the water in the migration chamber was found to be important, particularly when the larvae were large (Table VII). With the equipment used, a volume of 100 ml. was required, but migration was not improved greatly when the volume was in excess of this.

INHIBITION OF PHOTOMIGRATION BY INSECTICIDES

Attempts were made to use photomigration for the measurement of insecticidal action by adding large numbers of larvae to a series of dilutions of DDT and determining phototelotaxic response at the end of 24 hours. The slopes of the curves were steep and it proved difficult to select con-

centrations that would provide data in the 20 to 70 per cent inactivation range owing to day-to-day fluctuations in larval resistance. For example, in one experiment in which 200 to 300 larvae were used at each dose, only 0.5 per cent migrated at 0.050 p.p.m. DDT while 90 per cent migrated at 0.025 p.p.m.

It was noticed, however, that neural poisons produced disorientation and loss of ability to move away from a light source long before complete paralysis or death occurred. Therefore, a procedure for the determination of rate of inactivation was devised with the expectation that this property would be useful in bioassay.

Rate measurements at a standard concentration of insecticide were made by testing a number of groups of larvae at various times and by retesting the same group repeatedly. In the first method the readings were independent of one another and the viability of the larvae was not affected by previous migrations. However, the second method was used for obtaining the data presented here since it conserved material and could be more readily adapted to meet unknown situations.

Since the insecticide is absorbed by the larvae during the course of the test, the rate curves might be modified by changes in the absolute volume of the suspension or the number of larvae. A 100 ml. volume and 200 larvae were used in all cases to minimize these variations. The plastic migration tray was replaced by a glass trough to reduce losses by absorption on the equipment. In critical experiments the laboratory was maintained at $26^{\circ} \pm 2^{\circ}$ C. to eliminate effects from the temperature coefficient of the reaction. When more than 20 larvae were found to be inactivated during any time interval they were removed from the solution to facilitate the task of counting at the next reading. From data obtained in this way percentage inactivation in probit units was plotted against time. The curves were almost linear at high concentrations but at low concentrations became skewed (Fig. 4). This was to be expected, for even if the physiological resistance of the population were distributed normally, the concentration might be below the critical point for some individuals. Thus, if a population were treated with a dose that would ultimately produce 50 per cent inactivation the rate curve would have to approach this value asymptotically. This effect is shown most clearly in the case of pyrethrins (Table VIII). Immobilization proceeded very rapidly up to 26.5 minutes. Thereafter the rate decreased and became zero at a limiting value of 40 per cent. Evidently the toxicant produced its maximum effect early in the experiment and there was not sufficient material available to immobilize the remaining 60 per cent of the population.

The most useful value derived from the time-immobilization curve is the T_{50} , or time required to inactivate 50 per cent of the population. It is easily estimated by interpolation and has been used throughout this work

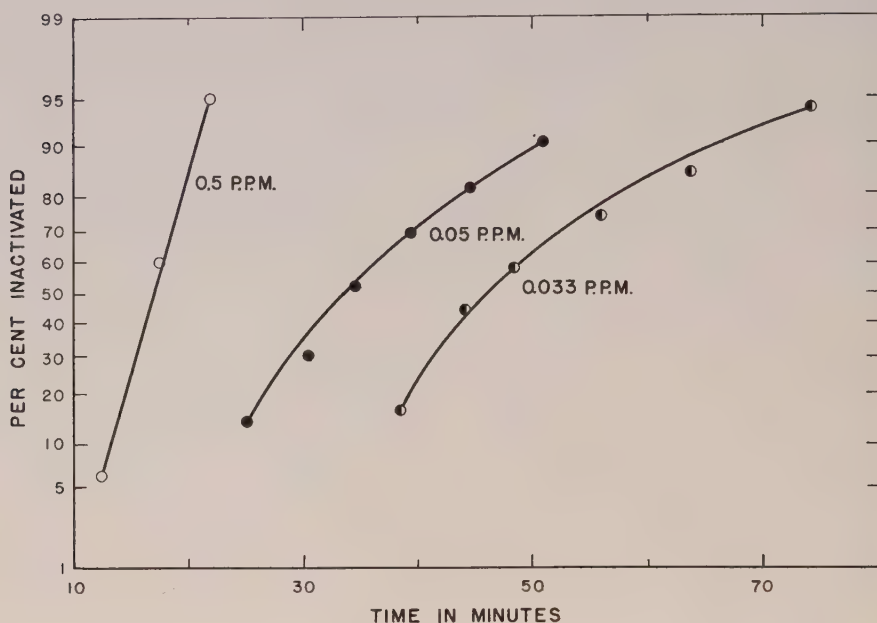


FIGURE 4. Rates of inactivation of groups of 200 mosquito larvae at various concentrations of DDT measured by loss of response to light.

as a criterion of toxicity. It can be determined on a single concentration of insecticide in from 5 to 200 minutes.

Low values for T_{50} appear to be characteristic of liposoluble neural and muscular poisons, so that interferences from accidental contaminants or toxic plant products are not serious. On the other hand, the rate of toxic action is slowed markedly by the presence of lipoids which tend to dissolve insecticide and reduce availability.

T_{50} values obtained on groups of 200 larvae withdrawn from the same population were reproducible within satisfactory limits. In a typical ex-

TABLE VIII

PERCENTAGE OF LARVAE INACTIVATED BY 0.01 P.P.M. PYRETHRINS AT VARIOUS TIMES

Time (minutes)	Percentage inactivated
6.5	11
15.5	20
19.5	25
26.5	34
30.5	37
40.5	40
58.5	40
92.5	40

periment the average value at a concentration of 0.1 p.p.m. DDT was 28.17 ± 1.71 minutes (Table IX). The age at which the larvae were removed from the culture medium was important. However, when washed free from traces of food they maintained reasonably constant resistance over a normal working day.

Before rearing methods were standardized, day-to-day reproducibility was unsatisfactory. T_{50} values at 0.125 p.p.m. DDT ranged from 27 to 105 minutes in experiments where larvae were raised on powdered dog biscuit without special attention to environmental conditions. However, better uniformity was obtained when food supply, temperature, and population density were accurately controlled. Under these conditions, T_{50} values at 0.1 p.p.m. DDT ranged from 19 to 35 minutes on a series of nineteen batches over a period of six weeks. The average value was 27.00 ± 4.21

TABLE IX
REPRODUCIBILITY OF THE T_{50} TEST ON LARVAE WITHDRAWN
FROM THE SAME POPULATION AT 0.1 P.P.M. DDT

Time after removal from culture medium (hours)	T_{50} (minutes)
0	31.0
0	26.5
1.0	27.0
1.0	29.5
1.5	27.5
1.5	27.5
Average	28.17 ± 1.71

minutes. Further improvements should result from studies on rearing now in progress.

Since acetone was used as the solvent for the insecticides, tests were made of its effect on response of normal larvae to light. Aqueous solutions were tested at concentrations ranging from 0.5 to 8.0 per cent to determine if the inclusion of solvent in the sample preparation scheme would be injurious. Up to a concentration of 2 per cent there was very little effect as evinced by a value of 5 per cent moribund at 25 minutes. At higher concentrations inactivation was quite rapid, but these levels would never be used in practice. Below 2 per cent there is little danger, providing reasonably active compounds are tested. However, with very slow-acting compounds, or with active compounds at high dilutions, the kill in the check would have to be taken into account.

T_{50} values at 0.1 p.p.m. DDT were generally determined on suspensions prepared by adding 1 ml. of a stock solution to 99 ml. water dropwise with swirling. Substantially equivalent results were obtained if the aqueous suspension was made up at higher concentrations and reduced to 0.1 p.p.m.

by serial dilution. When the acetone solution of DDT was placed in a flask and water added in inverse order the results were still the same indicating that variations in rate of kill were not caused by the method of dilution. This presumably could alter the particle-size distribution and hence the availability of the chemical. It is possible that the DDT was in true solution at some of the very low concentrations employed.

Glass apparatus was thoroughly cleaned to insure reproducibility of the results. The apparent toxicity of insecticide suspensions decreased greatly when permitted to stand in ordinary laboratory glassware from one-half to one hour before introducing the larvae. Evidently the insecticide dissolved in surface films on the glass and was no longer available to the larvae. This effect was minimized by treating all equipment with hot trisodium phosphate solution followed with sulfuric acid-dichromate cleaning solution prior to use. Absorption by the equipment was most serious in experiments run at low dilution where the inactivation time was long.

RELATIONSHIP OF CONCENTRATION TO TIME REQUIRED FOR INACTIVATION

When the initial concentration of insecticide was reduced, the T_{50} , or time required to inactivate 50 per cent of a population, increased. Data obtained over a limited range are conveniently interpreted by plotting the logarithm of T_{50} against the reciprocal of initial concentration (Fig. 5). If a high degree of accuracy is desired the reference points must be determined on larvae withdrawn from the same batch used for testing the unknowns. Otherwise the T_{50} at 0.1 p.p.m. DDT can be determined as a quick check and approximate adjustments made on the standard curves. This plot is not linear and on limited data is difficult to interpret unless more is known about the fundamental characteristics of the curve.

T_{50} values obtained on DDT in a concentration range of 0.0167 to 1.0 p.p.m. gave a hyperbolic curve when transferred to rectangular coordinates (Fig. 6). At high concentrations the rate of change of T_{50} with respect to concentration was small, while at low concentration it was large. From the standpoints of accuracy and convenience unknown solutions are best run within a 25- to 100-minute range. When the time is short, errors in estimating concentration will be large, while, when the time is long, the natural mortality of the larvae and the presence of foreign toxicants may tend to give misleading results.

The hyperbolic curve approaches asymptotes along the time and concentration axes, and these both have positive values. The asymptote parallel to the concentration axis represents the minimum time required for DDT to inactivate 50 per cent of a given population at the highest doses normally used. It probably owes its existence to the fact that the inactivation process consists of a series of consecutive steps in which the saturation

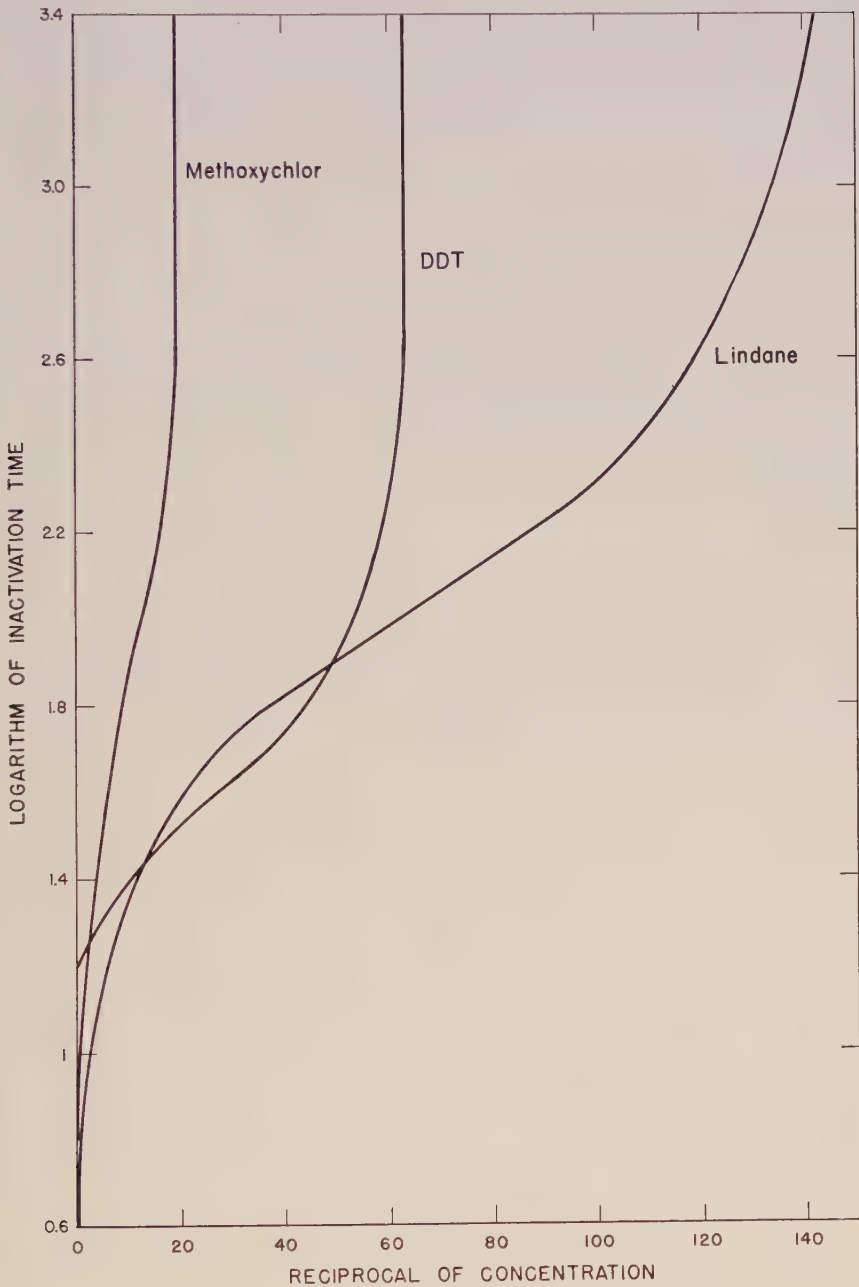


FIGURE 5. Relationship of logarithm of time required for inactivation of 50 per cent of a larval population toward light and reciprocal of initial concentration of insecticide calculated from equation (4).

concentration of the cuticle finally becomes limiting. Even if the absorbing tissues take up the maximum amount of insecticide immediately on contact with the suspension, a finite time would have to elapse before a critical amount of material could diffuse through the intermediate tissues and cause paralysis. This is arbitrarily called the minimum inactivation time and is designated by τ . At low concentrations the rates of cuticular absorption would be relatively slow and the time required for the build-up of a critical dose at the sites of action correspondingly long. In extremely dilute

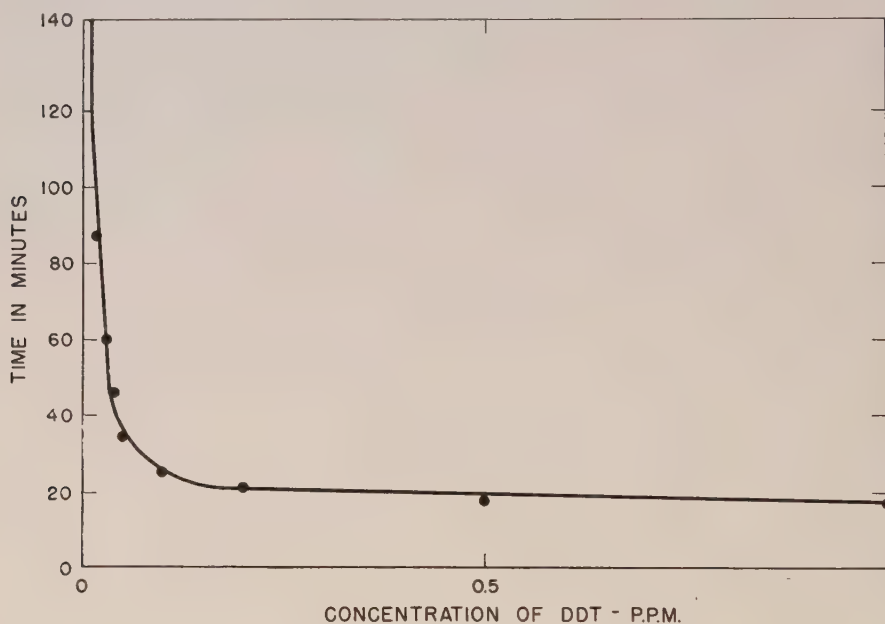


FIGURE 6. Relationship of concentration of DDT to time required for inactivation of 50 per cent of larval populations toward a standard photostimulus.

solutions the rate of absorption and translocation might finally become less than the rate of detoxification and permit indefinite survival of the larvae. The fact that finite volumes of insecticide are used must also be taken into account since the larvae probably absorb considerable amounts of the original material during the test. The rate of absorption from solution must, therefore, be an inverse function of time and probably follows a course similar to that of a first order reaction.

The asymptote parallel to the time axis represents the minimum concentration of toxicant that will inactivate 50 per cent of the population when the reaction time is unlimited. In practice it corresponds roughly to the LD₅₀ value obtained at 24 hours on the serial dilution test. It is referred to here as the critical concentration and designated by c_0 .

Data obtained on DDT (Table X) follow a modified first order rate equation with fair consistency when initial concentration and T₅₀ are regarded as the variables.

$$T_{50} = k \ln \frac{c}{c - c_0} + \tau$$

(3)

However, this equation is not satisfactory for methoxychlor and lindane and a more generally applicable relationship was sought. It was found that a modified equation for a hyperbola with positive asymptotes on the time

TABLE X

TIME REQUIRED TO IMMOBILIZE 50 PER CENT OF A LARVAL POPULATION (T₅₀) BY DDT, METHOXYCHLOR, AND LINDANE COMPARED WITH VALUES CALCULATED FROM EQUATION (4)

Concn. (p.p.m.)	DDT		Methoxychlor		Lindane	
	T ₅₀ observed	T ₅₀ calculated	T ₅₀ observed	T ₅₀ calculated	T ₅₀ observed	T ₅₀ calculated
1.0	17.3	17.3	6.5	6.8	5.3	5.5
0.5	18.1	18.1	12.3	12.3	9.3	8.5
0.2	22.0	20.8	32	29.6	15.5	15.1
0.1	25.0	25.1	67	68.8	24.3	23.7
0.08	—	—	104	99	—	—
0.05	34.0	34.2	—	—	38.5	38.2
0.033	45.5	45.1	—	—	52.0	51.8
0.025	55.0	60.3	—	—	—	—
0.020	88	87	—	—	96	80
0.0167	172	174	—	—	—	—
0.0100	—	—	—	—	200	199

and concentration axes would satisfy most of the data available. This takes the form

$$T_{50} = \sqrt{\tau^2 + \frac{A}{(c - c_0)^n}}$$

(4)

where *n* and *A* are constants, *τ* is the minimum inactivation time, *c*₀ the critical concentration, and *c* the initial concentration of the insecticide suspension.

Values for *τ* and *c*₀ are obtained by extrapolating the data at short times and low concentrations respectively. *A* and *n* are evaluated by plotting the logarithms of reduced concentration, *c* − *c*₀, against logarithms of a function of time, T₅₀² − *τ*². If care is exercised in selecting values for *c*₀ and *τ*, a straight line is obtained for most of the organic insecticides (Fig. 7). In this form *n* is the slope of the regression line and *A* an additive constant. The values for *n* and *A* are dependent upon the nature of the insecticide and the viability of the larvae used in the test.

This equation has no theoretical significance other than that it satisfies the requirement for asymptotes along the time and concentration axes. It is probable that it approximates a complex situation which is the resultant of a number of kinetic factors.

T_{50} values calculated from regression curves for DDT, methoxychlor,

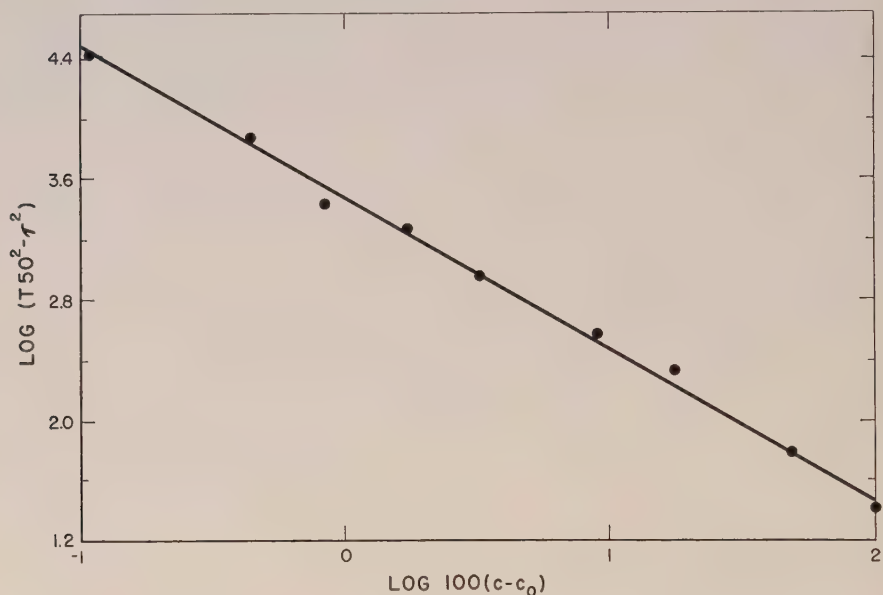


FIGURE 7. Relationship of a logarithmic function of concentration of DDT to a logarithmic function of the time required for 50 per cent inactivation of mosquito larval populations illustrating the correspondence of the experimental data with equation (4).

and lindane show good agreement with the experimentally determined values (Table X). Of course, the inclusion of four constants decreases the number of degrees of freedom, but in the cases of DDT and lindane there are a sufficient number of experimentally determined points to show that good correlation exists.

The shapes of the regression curves serve to define the inactivation characteristics of the various insecticides (Fig. 5). Thus, at high concentrations methoxychlor acts more rapidly than DDT but it does not hold up well on dilution. Lindane, on the other hand, acts extremely rapidly and is effective at very low concentrations. The constants τ and c_0 are useful for comparing the activities of various materials (Table XI). τ provides a measure of "knockdown" properties while c_0 is comparable to LD₅₀ and is a measure of ultimate toxicity. In general, pyrethrins, lindane, and methoxychlor are fast-acting, DDT is intermediate, while aldrin, heptachlor, and chlordan are relatively slow but persist at high dilutions. The

liposoluble organic materials as a group are more effective than the ionic insecticides. Thus, nicotine sulfate and sodium cyanide require concentrations of 10 to 100 p.p.m. for rapid inactivation while most of the organics are active at 0.01 to 1.0 p.p.m. This is probably caused by a more favorable partitioning between the aqueous medium and lipoidal tissues of the test organisms. These data are consistent with what is already known about the various materials but provide a more nearly quantitative measurement of the range of activity.

The accuracy with which an insecticide suspension can be assayed depends upon the material and the concentration at which it is tested, for the

TABLE XI

REGRESSION CURVE CONSTANTS FOR TYPICAL EXPERIMENTS ON VARIOUS INSECTICIDES

Constant	DDT	Methoxychlor	Lindane
τ (minutes)	16.4	1.5	0.6
c_0 (p.p.m.)	0.0156	0.044	0.007
n	1.02	1.65	1.24
A	28.8	40.7	29.5

error in concentration produced by a given error in T_{50} is approximately

$$\Delta c = \left(\frac{2 T_{50} A^{1/n}}{n(T_{50}^2 - \tau^2)^{1/n+1}} \right) \Delta T_{50} \quad (5)$$

In the case of DDT at 0.10 p.p.m. this is 0.02 p.p.m. assuming a standard deviation of 1.7 minutes in measurement of T_{50} . This relates only to the assay of pure suspensions and does not take into account losses in sample preparation or the presence of interfering materials.

BIOASSAY OF SPRAY RESIDUES

This paper is concerned primarily with the development and standardization of the photomigration test. Nevertheless some information has been obtained on extraction and sample preparation which shows the present limitations of the method for the bioassay of residues in food crops, and indicates what requirements must be met for satisfactory results.

Tressler (11) has shown that DDT added to processed foods can be recovered by extraction with benzene in a Waring blender. Work here indicates that pentane and hexane are more satisfactory solvents since they do not remove as large a quantity of plant material, and can be evaporated at lower temperatures. This latter factor is important since losses may occur during evaporation particularly when the insecticide residue is obtained in a more or less pure state. For example, Nolan and Wilcoxon (8) reported losses up to 20 per cent during the evaporation of parathion solutions, and even less satisfactory recoveries are obtained with DDT.

In one experiment carried out in this laboratory, 0.5 ml. of a 20 p.p.m. acetone solution of DDT was permitted to evaporate to dryness by standing in air at room temperature. When redissolved and assayed at a supposed concentration of 0.1 p.p.m. the T_{50} was 67 minutes. This represented a loss of 80 per cent of the original activity. On evaporating a similar solution in a current of air the loss was 86 per cent, but from petroleum ether it was reduced to 70 per cent. Recoveries were improved by use of low boiling solvents but the results were not dependable. Unfortunately solvent removal must be substantially complete since traces of hydrocarbons may produce temporary anesthesia of the larvae.

In view of the reproducibility of the T_{50} values it is difficult to explain these results on any basis other than volatilization. This problem must arise in other bioassay and chemical procedures when microgram quantities of chemical are recovered in a relatively pure state and spread out over large surfaces during removal of solvent. Lipids present in food extracts may act as carriers and reduce evaporation of the toxicant by providing a non-volatile residue from which diffusion and escape of toxicant molecules is slow. However, the addition of lipids would not be satisfactory since they inhibit larval inactivation.

It was considered possible that the addition of a high boiling solvent miscible with both acetone and water would reduce evaporation losses without interfering with the T_{50} determinations. To confirm this 0.5 ml. Carbitol (diethyleneglycol monoethyl ether—b.p. 195° C.) was added to 40 ml. of a solution of DDT in acetone and the solvent evaporated under a current of air. The residue was taken up in 1 ml. acetone and diluted to 100 ml. with water. The toxicity was equivalent to the standard, and the solvent alone had no effect on the larvae. However, Carbitol is not miscible with the aliphatic hydrocarbons and cannot be used in the evaporation of the original hexane extracts.

Even when the extraction of the food sample is satisfactory and evaporation losses are kept at a minimum, considerable depression of toxicity is caused by the presence of lipids. This was evident in several dilution tests on aldrin in the presence of cottonseed oil and phospholipids carried out by the method of Hartzell and Storrs (Table XII). It is even more serious in the T_{50} test.

In one instance the time required for 50 per cent inactivation by 0.1 p.p.m. DDT was increased from 22.5 minutes for the standard to 84 minutes for the same amount of DDT in the presence of the hexane extract from 20 g. processed spinach. Tests carried out in the presence of cocoa butter and emulsifying agents showed similar deviations. These were probably caused by the preferential solubility of the insecticide in the lipid phase. If the fat droplets are poorly distributed, or for any other reason cannot be completely assimilated by the larvae, apparent toxicity de-

creases. Therefore, interfering materials must be removed to obtain reliable results on microgram quantities of insecticide.

Carotenes, xanthophylls, and degraded chlorophylls are the major contaminants in the extracts from many processed foods. These are accompanied by fats, sterols, and cutinous waxes which also interfere. In many cases the plant pigments can be separated by chromatographic adsorption, but general procedures for the removal of unpigmented lipids have yet to be developed.

When the hexane extract from processed spinach containing 0.5 p.p.m. DDT was passed through a chromatographic column containing calcium carbonate the pigments were adsorbed in colored zones, and the lipids and insecticide were found in the percolate. The solvent was evaporated and

TABLE XII
REPRESSION OF TOXICITY OF ALDRIN TO MOSQUITO LARVAE
BY LIPIDS IN 24-HOUR SERIAL DILUTION TEST

Concn. (p.p.m.)	No. additives	Per cent mortality to larvae			
		Cottonseed oil		Phospholipid	
		0.01%	0.0025%	0.01%	0.0025%
0.5	100	95	90	95	100
0.25	100	70	90	85	100
0.125	100	65	60	75	95
0.063	80	55	45	75	70

the residue redissolved in acetone and suspended in water at a theoretical concentration of 0.1 p.p.m. DDT. The T_{50} was 54 minutes in comparison to 28 minutes for the standard. This is a decided improvement when compared to the results obtained on crude extracts, but experimental losses and repression of toxicity by residual waxes are still serious factors. Semiquantitative results in a toxicity range corresponding to 0.1 to 0.5 p.p.m. DDT can be obtained by comparison to suitably prepared standards, but the complete removal of interfering materials would have many obvious advantages. The procedure used here probably can be improved by further studies on adsorbents, conditions for elution, and prevention of losses through volatilization. However, the development of a process that would permit the separation of insecticides from the waxy residues will be required before results can be compared directly to a standard. Molecular distillation, countercurrent extraction, or adsorption of the insecticide on a material which will not retain fats and waxes appear to offer the best possibilities at the present time.

Interferences are less serious in the assay of spray deposits on unprocessed crops where the concentration of insecticide ranges from 2 to

50 p.p.m. This conclusion is based on bioassay results obtained on experimental spinach plots treated with commercial applications of DDT, methoxychlor, lindane, parathion, and aldrin. The foliage was sprayed to run off and the crop harvested the following day. Bioassays were made on aqueous suspensions of the extracts without prior chromatographic treatment. This would be unnecessary in any case since the tissues were not macerated and the extracts contained only cutinous waxes in addition to insecticide. The order of magnitude of the bioassay results compared favorably with relative spray concentrations (Table XIII). In the cases of DDT and parathion, parallel chemical analyses were carried out in the labora-

TABLE XIII
FIELD TRIAL ON THE APPLICATION OF THE BIOASSAY METHOD
TO RESIDUE ANALYSIS ON SPRAYED SPINACH

Spray treatment		Analysis (p.p.m.)		Dose ratio, DDT = 100	
Insecticide	Concn., oz./50 gal.	Bioassay	Chemical	Spray concn.	Bioassay
DDT	6	35	45	100	100
Methoxychlor	4	17	—	67	49
Lindane	3	15	—	50	43
Parathion	1.2	6.4	6.0	52	18
Aldrin	2	2.4	—	33	7

tories of the National Canners Association. Agreement was good when the results of the chemical analyses were corrected by factors based on the recovery of known amounts of insecticides added to extracts from untreated spinach.

Although T₅₀ results may be masked by the presence of lipids, the test is not as susceptible to errors caused by naturally-occurring plant poisons as is the serial dilution procedure. Rapid paralytic action is probably characteristic of specific neural and muscular poisons. The action of the toxicants found in plants is usually delayed longer and, while death may occur during a 24-hour incubation period, their action during the first half hour or so is frequently negligible. The crude extract from processed spinach at the rate of 20 g. of spinach per 100 ml. of final test solution caused only 5 per cent inactivation at the end of one-half hour, as compared to *ca* 50 per cent for 0.1 p.p.m. DDT. It can be assumed that if the lipid contents can be reduced to the point where they do not appreciably slow the action of the insecticides the presence of plant poisons will not present a serious problem.

PRACTICAL SIGNIFICANCE

The Federal Food, Drug and Cosmetic Act of 1938 (10) empowers the Federal Security Administrator to promulgate regulations limiting the

quantity of poisonous or deleterious substances in food. Up to the present, tolerances for insecticide residues have not been established, but current interest in possible health hazards arising from contamination make it likely that this will be done (6).

What the permissible limits will be remains to be seen. However, it is likely that methods will be required for the detection of toxic residues in a range of from 0.1 to 10 p.p.m. Concentrations higher than this will not normally be encountered if reasonable care is used in arranging spray schedules and removing residues. Based on present evidence, lower levels would not be significant toxicologically (9, 12) and may be impractical to maintain. For instance, in work carried out in this laboratory, it has been impossible to obtain potato extracts for use as standards with a toxicity equivalent to less than 0.02 p.p.m. chlordan. The potatoes were obtained from plots that presumably were untreated and the results were confirmed by chemical analysis. Evidently traces of toxicants can be picked up from the soil or during laboratory extractions, so to insist on a zero tolerance would discourage the use of many valuable materials without achieving anything by way of protecting the public.

Excellent chemical methods have been developed for the analysis of many of the commercially important pesticides. For others, the tests are time-consuming and not sufficiently sensitive. In every case the qualitative composition of the residue must be known before the appropriate analytical method can be selected. In crops contracted for from large acreages it is frequently possible to do this from a knowledge of spray schedules. There will be many instances, however, where the nature of the residue is unknown.

The mosquito larvae test is particularly suitable for preliminary bioassay since it is sensitive to a wide variety of organic insecticides at low concentrations. Furthermore, relative toxicity parallels mammalian toxicity to some extent so it will selectively detect the more hazardous residues. After the initial test, samples which exhibit high toxicity could be analyzed by chemical methods and the remainder passed as safe for processing.

The photomigration method has an advantage in that results can be obtained within a few hours after the sample has been extracted and purified. When adequate separation procedures are perfected, toxicity data should be available on the same day the sample is submitted. Objective classification and ability to handle large populations serve to augment the reliability of the results, and a rapid check on larval viability with reference to a standard should prevent large numbers of tests from being carried out with unhealthy populations.

It may be unnecessary to obtain complete time-inactivation curves in all cases. Minimum inactivation time varies widely, but, for all of the insecticides studied, the T₅₀ at 0.1 p.p.m. was less than 100 minutes with the

exception of ionic materials such as nicotine sulfate and sodium cyanide. It would be possible, therefore, to make a single reading at a pre-set time in order to decide whether further analysis is indicated. If less than 50 per cent of the population were inactivated in the pre-set time, it would be concluded that insecticide residues were not present in dangerous amounts. Where T_{50} values are actually obtained, results could be recorded in toxicity units of DDT.

The above system makes the assumption that insecticides can be recovered free from interfering lipids. This is not the case at present although there is basis for the hope that the techniques can be improved. An interim solution would be the use of correction factors based on the bioassay of known amounts of insecticide in the presence of extracts from various food crops. This would reduce accuracy and sensitivity and require a large number of standards. Continued work on improved techniques for separation appears to be the best alternative.

Aside from limitations in the removal of interfering lipids, the photomigration test appears to be satisfactory in its present form. Automatic timing and electromagnetic control of the barriers would improve accuracy and ease of manipulation somewhat, but are not essential when small numbers of samples are handled. Some consideration has been given to automatic counting by photoelectric or radioactive tracer methods, but direct count is probably easiest once the larvae have been segregated.

Day-to-day reproducibility of larval resistance is satisfactory for bioassays in which only the order of magnitude of toxicant is desired. For higher accuracy or in fundamental studies on the mechanism of inactivation, a greater degree of uniformity would be helpful. This can probably be achieved by genetic selection for desirable characteristics and rigorous control of environmental conditions. Temperature, microbiological contamination, and nutritional balance are all important and warrant further study. The use of species which can overwinter in the larval stage such as *Anopheles claviger* Meig. (2, p. 40) may be worth consideration. It might be possible to rear and standardize large populations and maintain them in a dormant condition over long periods of time. This should provide a population with relatively constant characteristics on which to draw for test purposes.

Thus far, experimental work has been carried out only on larvae of *Aedes aegypti* (L.). In regions where quarantine restrictions make it impossible to maintain this species, culicine or anopheline larvae could probably be substituted. The range of toxicological activity available for study might be broadened by including free-swimming larvae outside of the Order Diptera. In all these cases the use of an objective method for assessing toxic action should serve to minimize the errors inherent in bioassay.

In addition to its applications in bioassay, the photomigration tech-

nique may have some value for the secondary screening of new insecticidal materials. A standard mortality test at high concentrations is satisfactory for separating active from inactive compounds, but gives little additional information. T₅₀ determinations made on a dilution series should serve to establish "knockdown" characteristics and ultimate toxicity with relatively little effort. In this way the entire range of action against motile larval forms can be studied. There is reason to believe the method would be useful in studies of synergism, antagonism, absorption rates, and various physico-chemical processes involved in insect toxicology. Further information on time-concentration relationships and the role of lipids and surface active materials should provide a better understanding of inactivation mechanisms.

SUMMARY

A new method for the bioassay of insecticides is described which is based on the negative phototaxic response of larvae of *Aedes aegypti* (L.). The larvae are confined behind a porous barrier in a shallow glass trough containing a dilute aqueous suspension of insecticide. When a 500-watt light source parallel to the longitudinal axis of the trough is turned on and the barrier removed, viable larvae rapidly migrate to the far end. After an exposure of one minute, a second barrier is dropped into place and the larvae that are trapped behind it are regarded as moribund.

The technique was used successfully to determine paralytic action in serial dilution tests, and to measure T₅₀, or the time required to inactivate 50 per cent of the population. The method is semi-specific for liposoluble neural poisons and narcotics, and can be used in the secondary screening of new insecticides. Knockdown characteristics, persistence on dilution, and other toxicological characteristics can be compared to a standard on a quantitative basis.

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SEED STORAGE AND VIABILITY

LELA V. BARTON

Early results of most of the storage experiments to be described in this paper have been reported previously (1, 2, 3, 4, 5, 7, 8). Since those reports, additional data have thrown some light on the physiological behavior of the seeds concerned and have yielded valuable information for seedsmen and foresters. The life span of some tree, vegetable, and flower seeds under different storage conditions will be given. The superiority of below-freezing storage as compared to low temperatures above freezing and the advantage of sealed storage will be demonstrated. Emphasis will be placed on the importance of the method used to test germination capacity and vigor.

MATERIAL AND METHODS

The tree seeds used were: *Abies grandis*, *A. nobilis*, *A. magnifica shastensis*, *Pinus taeda* L., *P. caribaea* Morelet, *P. echinata* Mill., *P. palustris* Mill., *P. resinosa* Ait., *Picea excelsa* Link, *P. canadensis* (Mill.) BSP, *Ulmus americana* L. Vegetable seeds included in the study were: carrot (*Daucus carota* L. var. *sativa* DC.), eggplant (*Solanum melongena* L.), lettuce (*Lactuca sativa* L.), onion (*Allium cepa* L.), pepper (*Capsicum frutescens* L. [*C. annuum* L.]), and tomato (*Lycopersicon esculentum* Mill.). Flower seeds were: aster, Queen of the Market (*Callistephus chinensis* Nees), *Verbena teucroides* Gill. & Hook., Royal Bouquet mixed, *Lilium regale* Wilson, *Gladiolus* sp., tree peony (*Paeonia suffruticosa* Andr.), annual and perennial *Delphinium*. Seeds of cotton, Egyptian (*Gossypium* sp.) and dandelion (*Taraxacum officinale* Weber) were also used.

Details of seed source and manner of storage may be found in previous publications (1, 2, 3, 4, 5, 7, 8) for many of these seeds. However, this is the first report of storage tests of some of the seeds as follows: *Gladiolus* seeds were received from The Research Committee of the Maryland Gladiolus Society, through the courtesy of Mr. Fred W. Gray; cotton seeds were from the United States Department of Agriculture; *Abies* seeds were from the Columbia National Forest; and tree peony seeds were collected from the grounds of Boyce Thompson Institute.

In general, 100 seeds each were used in soil plantings in the greenhouse to test the germination capacity of the tree seeds; duplicates of 100 seeds each in ovens at controlled temperatures and the same number of seeds in soil plantings in the greenhouse served for testing vegetable, flower, dandelion, and cotton seeds. Seedling emergence from the soil plantings was somewhat less than germination counts on filter paper, but the latter

had the advantage of being constant from one testing period to the other, while greenhouse temperatures varied.

In the tables to be presented, dashes indicate that the seeds had lost their viability as shown by previous tests, or that the seed supply was exhausted, while zeros show germination failures.

RESULTS

CONIFER SEEDS

Abies. Seeds of the three species, *A. grandis*, *A. nobilis*, and *A. magnifica shastensis*, were of the 1926 crop and were placed in special storage on March 7 and May 19, 1927. Up to the time of special storage, they had been held at ordinary room temperatures. Moisture contents at different levels were secured by storing the seed lots over different concentrations of sulfuric acid for six weeks after which they were removed from the desiccators and placed in bottles fitted with paraffined corks. Storage was in the laboratory and at low temperature. Low temperature storage was at approximately 8° C. for the first nine years after which the seeds were transferred to a room at approximately -4° C. The only seeds surviving laboratory storage for as long as one year were those of *A. nobilis* containing 11 and 13 per cent moisture and *A. magnifica shastensis* containing 18 per cent moisture. Lower (down to 7 per cent) and higher (up to 29 per cent) moisture contents were detrimental to keeping quality in the laboratory.

At the low temperature storage, *Abies* seeds remained viable much longer (Table I). Seedling production in the greenhouse after one month's pretreatment in moist granulated peat moss at 5° C. was the criterion for viability. The germination obtained at the time of storage (0 years), i.e.

TABLE I
VIABILITY OF ABIES SEEDS STORED IN SEALED CONTAINERS
AT LOW TEMPERATURE

Species	Moisture content, %	Per cent seedling production after storage for years								
		0	1	2	9	10	11	14	15	16
<i>A. grandis</i>	9	58	44	27	27	34	24	19	2	17
	11	98	24	15	57	45	46	36	3	26
	12	54	38	2	9	29	22	4	0	6
	15	64	36	13	19	14	15	9	5	8
<i>A. nobilis</i>	7	50	36	14	36	15	17	7	—	—
	8	62	44	42	46	54	31	22	12	30
	11	66	34	32	54	29	42	31	20	33
	13	52	34	33	46	42	43	29	15	22
<i>A. magnifica shastensis</i>	8	20	20	12	28	32	19	10	—	—
	11	38	30	14	31	33	37	9	5	12
	13	32	26	14	30	30	20	23	6	19
	18	36	32	20	29	55	20	7	2	9

after six weeks in a desiccator over sulfuric acid for moisture adjustment, indicated the harmful effect of low and high moisture contents. This was especially noticeable for *A. grandis* where lowering the moisture to 9 per cent or raising it to 12 or 15 per cent decreased germination from the 98 per cent exhibited by seeds with 11 per cent moisture to 54 and 64 per cent. These effects are also indicated by the resistance of the various lots to further storage. In spite of the effort to standardize the testing procedure, a great variation in germination capacity from year to year was apparent.

TABLE II
SEEDLING PRODUCTION IN THE GREENHOUSE FROM PINUS SEEDS STORED
AIR-DRY UNDER VARIOUS CONDITIONS

Species*	Storage		Per cent seedling production after storage for years															
	Temp., ° C.	Open (O) or sealed (S)	Planted directly from storage								Pretreated at 5° C. for 1 month							
			8	9	10	11	12	13	15	16	8	9	10	11	12	13	15	16
<i>P. echinata</i>	Lab.	S-vac.	34	14	14	10	9	8	10	6	25	10	18	17	19	20	0	0
	5	O	23	11	17	—	1	0	1	0	53	14	14	—	6	3	1	3
		S-air	8	17	31	—	15	8	18	10	35	16	39	—	50	38	36	16
	—4	S-vac.	45	44	60	—	45	40	50	48	72	53	67	—	78	54	61	36
		O	34	32	63	23	29	28	26	37	55	60	64	82	78	74	64	36
	—4	S-air	24	33	27	11	27	21	8	27	41	16	39	48	41	49	35	42
		S-vac.	52	51	54	39	53	32	49	30	85	77	83	67	84	84	73	68
<i>P. taeda</i>	Lab.	S-vac.	16	12	27	15	5	5	13	—	35	34	30	19	33	43	22	—
	5	O	24	4	19	—	1	0	1	—	57	42	49	—	15	17	3	0
		S-air	18	3	17	—	7	3	8	1	49	25	41	—	13	28	15	3
	—4	S-vac.	31	8	22	—	19	6	13	—	73	58	74	—	69	68	71	—
		O	25	9	19	12	—	—	—	—	90	70	88	76	80	—	—	—
	—4	S-air	45	4	11	3	12	1	—	—	78	48	69	51	73	74	—	—
		S-vac.	55	1	21	2	4	4	—	—	87	66	89	74	83	87	89	—

* At the beginning of the storage experiment, *P. echinata* and *P. taeda* gave up to 84 per cent seedling production in the greenhouse.

However, there is no doubt that low temperature markedly prolongs the life of *Abies* seeds.

Pinus. Seeds described in the publication of 1935 (2) were tested further after various storage periods until they were no longer capable of germination or until the seed supply was exhausted. The data in Tables II and III will suffice to show the trend of results secured from all of these seeds.

A few effects are outstanding. Of the three temperatures tried, that of the laboratory resulted in more rapid deterioration than 5° C., which latter temperature was, in turn, less favorable than -4° C. for seeds of *P. echi-*

nata and *P. taeda* (Table II). Seeds stored in reduced air pressure obtained by means of a vacuum pump (vac.) kept better than those sealed in air or without sealing. This was especially to be seen in laboratory storage where seeds in evacuated flasks maintained some viability up to 15 years of storage. This is in contrast to open or air-sealed storage at this temperature where very few seeds survived for two or three years. The data in Table II also point to the advantage of pretreating these pine seeds in moist granulated peat moss for one month prior to planting in soil in the greenhouse. This is especially marked for *P. taeda* seeds. The results shown in Table II are those from seeds reported in Experiment 1 in 1935 (2).

TABLE III
SEEDLING PRODUCTION IN THE GREENHOUSE FROM CONIFER
SEEDS STORED AT APPROXIMATELY -4° C.

Species*	Wt. of seeds +CaO, g.	Per cent seedling production after storage for years													
		Planted directly from storage							Pretreated at 5° C. for 1 month						
		8	9	10	11	12	14	15	8	9	10	11	12	14	15
<i>Pinus taeda</i>	100 + 0	0	1	2	0	0	—	—	44	40	46	64	55	—	—
	100 + 10	1	1	2	0	0	—	—	50	31	40	57	38	59	—
	100 + 17	0	0	0	1	0	3	1	53	30	29	66	39	51	45
	100 + 25	0	2	0	0	2	15	0	64	36	27	53	50	58	34
	100 + 32	0	1	0	0	5	3	—	48	21	20	52	46	41	—
	100 + 45	0	0	0	0	0	0	—	14	5	2	0	1	0	—
<i>Picea excelsa</i>	50 + 0	51	57	37	48	43	44	40	56	33	51	41	45	14	9
	50 + 8	33	29	23	24	27	23	37	21	37	14	17	16	2	0
<i>Picea canadensis</i>	25 + 0	42	58	36	43	61	40	33	63	74	79	80	84	34	40
	25 + 4	34	19	15	17	38	18	1	70	53	63	63	67	12	8
	25 + 8	7	1	0	0	3	0	0	19	9	3	6	4	0	0
	25 + 15	3	2	1	1	4	0	0	20	11	4	7	12	1	0

* At the beginning of the storage experiment *Pinus taeda*, *Picea excelsa*, and *Picea canadensis* gave up to 52, 65, and 48 per cent seedling production respectively.

This experiment also included seeds of *P. caribaea*, *P. resinosa*, and *P. palustris* not included in this table. The short seed supply of these species curtailed further testing.

Mixing pine seeds with different amounts of calcium oxide to attain different moisture contents for storage has the advantage of being a very simple and effective procedure but care must be taken to avoid excess drying. Seeds of *P. taeda* so stored for 8 to 15 years in a room at approximately -4° C. showed the definite ill effects of too much drying (Table III). Again, the necessity for pretreatment of the seeds for seedling production in the soil in the greenhouse is demonstrated. Seeds of *P. palustris* mixed with small amounts of calcium oxide retained their germination capacity for nine years at -4° C.

Picea. *P. excelsa* and *P. canadensis* can be kept in sealed containers at approximately -4° C. for as long as 15 years provided they are not dried too much (Table III). It will be noted that the germination of neither of these forms was dependent upon pretreatment in a moist medium at 5° C. On the contrary, such pretreatment tended to decrease germination in some cases.

ELM

An earlier publication from this laboratory reported the successful storage of seeds of *Ulmus americana* for as long as 16 months in sealed containers at 5° C. or below (4). Up to that time (1939), 10½ months had

TABLE IV
SEEDLING PRODUCTION IN THE GREENHOUSE FROM ELM SEEDS
STORED UNDER VARIOUS CONDITIONS

Germination pretreatment	Storage			Seedling production after storage for years						
	Temp., ° C.	Moisture content, %	Container	2	4	6	8	10	12	15
None	Lab.	3	Sealed	12	3	0	—	—	—	—
		2	Sealed	13	1	0	—	—	—	—
	5	7	Sealed	17	11	19	10	0	0	—
		3	Sealed	29	14	24	14	5	0	—
		2	Sealed	16	12	9	23	8	0	—
	-4	—	Open	10	14	4	10	4	0	—
		7	Sealed	14	22	15	16	26	15	7
		3	Sealed	22	40	22	33	29	13	12
		2	Sealed	17	43	18	18	22	18	—
1 Month at 5° C.	Lab.	7	Sealed	70	2	—	—	—	—	—
		3	Sealed	55	33	0	—	—	—	—
		2	Sealed	58	11	0	—	—	—	—
	5	7	Sealed	90	75	78	15	2	0	—
		3	Sealed	82	84	73	51	25	1	—
		2	Sealed	82	83	55	31	18	0	—
	-4	—	Open	78	58	51	15	14	1	—
		7	Sealed	91	70	78	44	67	59	45
		3	Sealed	87	57	73	83	62	83	75
		2	Sealed	80	46	80	64	68	72	—

been given as their maximum life span, though later (1946) some germination after one year's storage was reported by Johnson (18). Some of the elm seeds stored originally in June, 1937, were still viable 15 years later (Table IV). Again in this table and in Figure 1 is shown the necessity for pretreatment of stored seeds to obtain a true index of their germination capacity. A comparison of the seedling production obtained in the greenhouse without pretreatment and after pretreatment in moist granulated

peat moss at 5°C . for one month makes this clear. As has already been pointed out (4), good germination is also to be had in soil in the greenhouse after soaking the seeds under a light source for 24 hours before planting, or by mixing them with moist granulated peat moss and germinating them at a daily alternating temperature of 10° to 25°C . In the discussion of storage effects below, seedling production in soil in the greenhouse following pretreatment at 5°C . will be considered.

Air-dry seeds, i.e. containing 7 per cent moisture at the time of storage, were stored in open as well as in sealed containers in the laboratory and

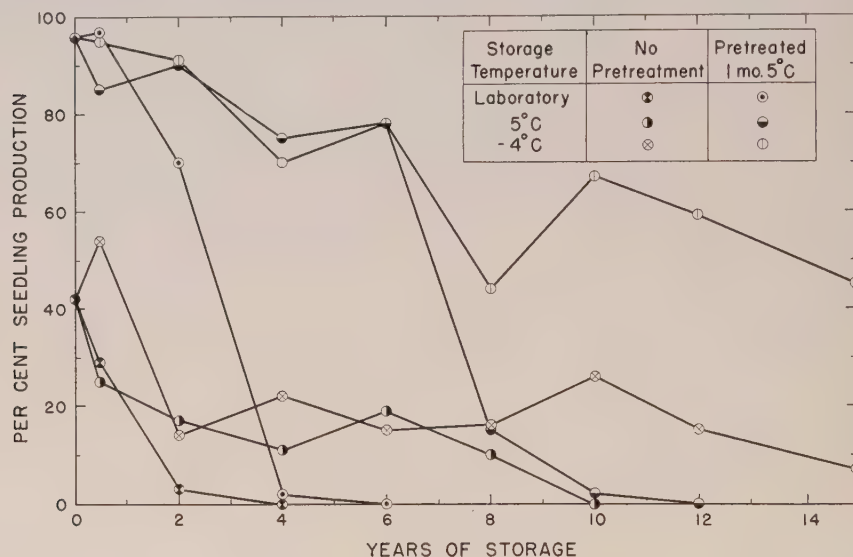


FIGURE 1. Effect of pretreatment for one month at 5°C . on seedling production in the greenhouse from air-dry elm seeds stored in sealed containers at different temperatures.

at 5°C ., but are not shown in the table because they did not survive 24 months of such storage. Seeds in open containers at -4°C . fared much better, germinating up to 51 per cent after 72 months of storage. Seeds dried to 3 or 2 per cent moisture remained viable longer regardless of temperature. As to the effect of temperature itself, there is no doubt of the superiority of -4°C . over laboratory or 5°C . for maintaining viability. Safe storage in the laboratory did not exceed 24 months. At 5°C . the seeds could be stored for at least 72 months, and at -4°C . the life span has already been extended to 180 months. There is still a small supply of seeds to make future tests. The extension of the life of elm seeds by controlled storage conditions is noteworthy in view of their reported short life span.

VEGETABLE SEEDS

The effect of storage for 1 to 6 years on the viability of vegetable seeds is the subject of two publications from this laboratory (1, 3). Data are now

available from tests using these same seeds for periods up to 20 years of storage. Storage was at two temperatures, laboratory and -4°C . Results from germination tests at controlled temperatures after 8 to 20 years are shown in Table V. Germination temperatures used were: constant 25°C .

TABLE V

GERMINATION OF VEGETABLE SEEDS ON MOIST FILTER PAPER AT CONTROLLED TEMPERATURES AFTER STORAGE UNDER VARIOUS CONDITIONS

Seed	Storage		Per cent germination after storage in laboratory for years								Per cent germination after storage at -4° C. for years							
	Con- tainer	Moisture content, %**	8	10	12	14	16	18	20	8	10	12	14	16	18	20		
Carrot 67*	Open	—	46	22	2	—	—	—	—	66	60	62	73	64	60	63		
	Sealed	10.7	—	—	—	—	—	—	—	69	68	68	69	74	66	65		
		a	62	59	56	54	43	10	2	74	64	69	70	68	65	63		
		b	57	65	54	50	35	6	1	71	65	65	74	73	68	65		
	c	65	64	52	55	48	22	6	69	76	66	74	66	66	69			
Eggplant 86*	Open	—	59	27	17	3	—	—	—	79	76	—	—	—	—	—		
	Sealed	10.4	37	4	0	2	—	—	—	86	79	84	81	—	—	—		
		a	75	78	80	50	—	—	—	83	77	76	—	—	—	—		
		b	80	72	66	75	54	30	10	84	88	83	88	82	81	86		
	c	78	79	84	83	77	54	42	87	84	83	88	88	80	86			
Lettuce 98*	Open	—	—	—	—	—	—	—	—	94	93	93	88	95	89	88		
	Sealed	8.2	—	—	—	—	—	—	—	96	96	87	84	81	81	86		
		a	69	61	67	—	—	—	—	93	85	80	83	77	76	80		
		b	66	64	1	—	—	—	—	93	94	83	80	82	80	76		
	c	84	73	3	22	0	—	—	95	91	88	81	85	76	84			
Onion 98*	Open	—	—	—	—	—	—	—	—	85	69	—	—	—	—	—		
	Sealed	12.5	—	—	—	—	—	—	—	85	71	—	—	—	—	—		
		a	62	19	—	—	—	—	—	94	90	—	—	—	—	—		
		b	33	2	1	—	—	—	—	96	87	89	88	88	79	—		
	c	76	71	32	3	—	—	—	96	92	95	92	87	89	93			
Pepper 73*	Open	—	—	—	—	—	—	—	—	69	59	62	69	57	32	19		
	Sealed	10.4	—	—	—	—	—	—	—	72	65	73	64	64	—	—		
		a	1	1	0	—	—	—	—	74	72	61	65	72	73	54		
		b	4	0	0	—	—	—	—	66	76	71	72	70	71	67		
	c	39	20	1	—	—	—	—	63	76	70	66	62	65	61			
Tomato 93*	Open	—	48	27	3	3	—	—	—	86	92	88	—	—	—	—		
	Sealed	10.0	0	—	—	—	—	—	—	91	84	—	—	—	—	—		
		a	80	75	—	—	—	—	—	91	90	—	—	—	—	—		
		b	90	84	75	74	45	16	1	92	95	91	94	90	—	—		
	c	86	83	79	77	71	49	23	90	97	92	91	92	97	88			

* Per cent germination of seeds at beginning of storage.

** At the beginning of the storage period.

a = Dried over CaO to remove about $\frac{1}{3}$ of indicated moisture; b = mixed with CaO to remove about $\frac{1}{3}$ of indicated moisture; c = mixed with CaO to remove about $\frac{1}{2}$ of indicated moisture.

for onion, pepper, and tomato; daily alternation of 15° to 30° C. for carrot and lettuce; and daily alternation of 20° to 30° C. for eggplant.

Storage periods up to six years had shown tomato and eggplant seeds most resistant to laboratory temperature. Lettuce and onion seeds were least resistant and carrot and pepper seeds were intermediate in their response to this temperature. Further tests have revealed that carrot, eggplant, and tomato were still capable of about 50 per cent germination after open storage for eight years in the laboratory (Table V). The life span was extended by drying the seeds before sealing. Drying was achieved by placing the samples over calcium oxide in a desiccator or by mixing the seeds directly with weighed amounts of calcium oxide, after which the lots were placed in sealed containers.

Though some vegetable seeds remained viable for several years at laboratory temperature, -4° C. proved far superior for keeping the seeds (Table V). Even onion seeds, the germination capacity of which is seriously impaired after one year in open storage in the laboratory, gave 93 per cent germination after 20 years, when they were mixed with calcium oxide and placed in sealed containers at -4° C.

DANDELION SEEDS

Seeds were stored at three temperatures, laboratory, 5° C., and -4° C. Air-dry seeds at the time of storage contained 7.9 per cent moisture. Some samples were stored with this moisture content, while other samples of the same seed lot were adjusted by storing in desiccators over calcium oxide to 6.2, 5.0, and 3.9 per cent moisture before storage in sealed containers. Sealing was accomplished in two ways. In one case approximately 400 seeds each were placed in small glass tubes hermetically sealed in air and in vacuum, and in the other case, larger lots were placed in tin cans with tight-fitting lids sealed with sealing wax. In the latter case each can was opened and samples taken out for testing, after which the cans were resealed. Thus the seeds remaining after each germination test had been subjected to fluctuation in moisture and gaseous exchange attendant upon the opening of the can, a condition which did not exist for individual testing samples stored in glass tubes. That the method of sealing proved important in keeping quality has been shown in a previous article (5).

Further effects are shown in Figure 2 which depicts the results of extended storage up to 16 years of seeds with initial moisture contents of 7.9 and 3.9 per cent. Seeds with 7.9 per cent moisture (Fig. 2 A) stored in a tin can in the laboratory lost germination capacity completely within two years, while those stored in sealed glass tubes still gave 66 per cent germination at that time. Furthermore, 12 per cent of the latter germinated after four years' storage. An even more striking difference in the two sealing methods is to be seen in the 5° C. storage. In tin cans high germination

capacity was retained for four years, after which there was rather rapid deterioration up to 10 years, when only 7 per cent of the seeds were alive. In glass tubes at 5° C., on the other hand, full viability was retained for 14 years at which time the seed supply was exhausted. Even at the more favorable storage temperature of -4° C. the advantage of the glass tube over the tin can for keeping these seeds is evident though the break in germination representing the beginning of deterioration in this case was delayed until after 12 years of storage.

The life span of dandelion seeds may be extended by drying to 3.9 per cent moisture before storage as shown in Figure 2 B. In this case, de-

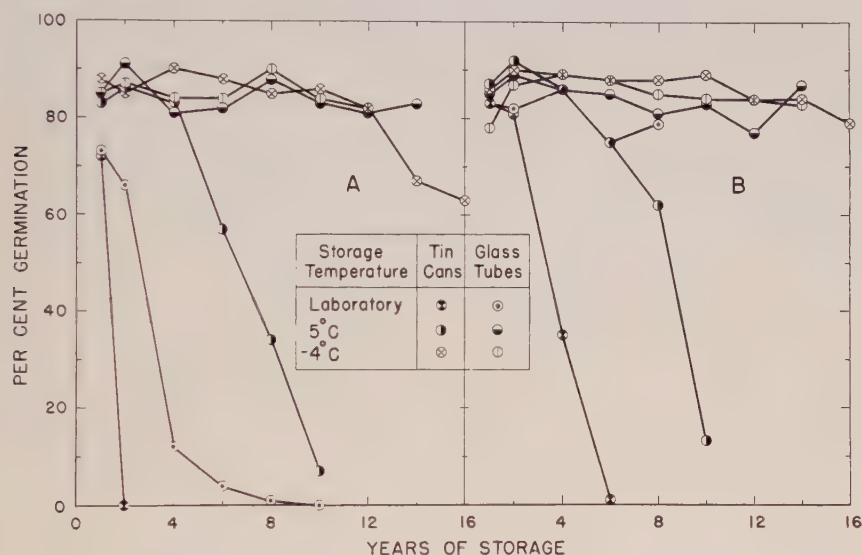


FIGURE 2. Effect of method of sealing and temperature on the viability of dandelion seeds stored with (A) 7.9, and (B) 3.9 per cent moisture. Germination at time of storage was 91 per cent.

terioration in a tin can in the laboratory became marked only after 4 years of storage but the favorable effect of storing in glass tubes is still evident. Similar effects are to be seen for 5° C. storage, but were not evident for -4° C. storage up to 16 years. Some implications of these effects will be given under "Discussion."

FLOWER SEEDS

Aster and Verbena. Seeds of both of these species were stored in the same manner as dandelion, except that the moisture contents were as shown in Table VI. In this table the germination of seeds from tin can storage is given. Glass tube samples were exhausted after 73 months of

storage. From the beginning of storage up to 73 months, glass tubes were better than tin cans for maintaining viability.

Aster seeds kept well for 192 months when they were sealed in tin cans and stored at -4°C . regardless of the initial moisture content of the seeds (Table VI). Also, within the limits of this experiment, the moisture content was without effect when the storage was at 5°C . where a rapid decline in germination capacity was evident after 132 months. The effect of open storage is given for -4°C . only, since seeds in open containers in the

TABLE VI

GERMINATION OF ASTER AND VERBENA SEEDS ON MOIST FILTER PAPER AT CONTROLLED TEMPERATURES AFTER STORAGE IN TIN CANS UNDER VARIOUS CONDITIONS

Seed	Storage			Per cent germination after storage for months						
	Con- tainer	Moisture content, %	Temp., ° C.	49	85	108	132	149	174	192
Aster	Open	—	—4	82	78	79	66	71	47	21
	Sealed	7.9	5 —4	88 89	81 86	79 87	36 75	14 86	0 85	— 89
		6.7	5 —4	86 88	80 90	75 87	23 77	0 93	0 86	— 75
		4.6	5 —4	86 81	82 89	77 84	27 75	1 87	0 87	— 81
	Open	—	—4	23	7	17	9	7	5	5
Verbena	Sealed	6.2	5 —4	39 25	14 13	20 22	16 18	9 31	0 37	— 32
		5.8	5 —4	24 24	9 15	16 28	8 22	1 21	0 23	— 24
		4.2	5 —4	21 19	14 18	18 27	11 16	4 31	0 27	— 20

humid 5°C . room had decreased in germination after one year and declined rapidly thereafter. These effects along with those of laboratory storage have been given in a previous paper (5). Both aster and *Verbena* seeds can be stored for more than 16 years at -4°C . when the moisture content does not exceed 7.9 and 6.2 per cent respectively (Table VI). Germination of fresh aster seeds was 91 per cent; fresh *Verbena* seeds 45 per cent.

Gladiolus. Seeds have been stored in this laboratory and tested annually for 12 years. Germination tests were conducted at 20°C . in a medium of granulated peat moss. Eighty-two per cent germination was secured from these seeds at the time of storage.

It will be seen from Table VII that air-dry seeds were stored in open and sealed containers in the laboratory and at 5° and -4°C . The moisture

content of some of the seeds was reduced to 5.8 per cent by drying over calcium oxide after which they were placed in sealed containers at the three temperatures. Sealing was in tin cans with tight-fitting lids covered with sealing wax. Once again, sealed storage was more effective than open storage; 5° C. better than laboratory; and -4° C. better than 5° C. for keeping the seeds viable. Also, more rapid deterioration resulted from open storage in a very humid 5° C. room than in the laboratory. This has been noted repeatedly for many kinds of seeds. For the first 10 years all germination

TABLE VII
GERMINATION OF GLADIOLUS SEEDS IN MOIST GRANULATED PEAT MOSS AT
20° C. AFTER STORAGE UNDER VARIOUS CONDITIONS

Moisture content, %	Storage		Per cent germination after storage for years					
	Temp., ° C.	Container	2	4	6	8	10	12
7.6	Lab.	Open	62	16	0	—	—	—
		Sealed	70	65	49	13	0(4)*	0(0)*
	5	Open	14	0	—	—	—	—
		Sealed	80	81	72	4	0(88)*	0(65)*
	-4	Open	61	59	44	9	4	0
		Sealed	80	79	86	87	82(92)*	73(76)*
5.8	Lab.	Sealed	83	76	66	12	0(11)*	1(2)*
	5	Sealed	82	91	85	35	0(80)*	0(77)*
	-4	Sealed	72	87	93	74	73(94)*	70(81)*

* From can opened for the first time after 10 years of storage.

samples were removed from the same sealed tin can (*a*) after which the can was resealed for further storage. At the 10-year period, the second storage can (*b*) which had never been opened from the beginning, was opened and the germination of the seeds compared with that of can *a*. The difference in germination capacity of seeds from cans *a* and *b* (Table VII) emphasizes the advantage to be gained by the prevention of fluctuating storage conditions as mentioned above for dandelion, aster and *Verbena*.

For *Gladiolus*, successful storage for 12 years at -4° C., or at 5° C. if the container is not opened in the interim, is indicated.

DISCUSSION

Temperature. Temperature of storage is a factor of vital importance in determining the life span of seeds. It has been known for a long time that low temperature is more effective than higher temperature, but temperatures above freezing, especially between 5° C. and freezing, have been

considered adequate. The fact is, however, that a very moist atmosphere at 5° C. brings about more rapid deterioration than ordinary laboratory conditions in the region of Yonkers, N. Y. This emphasizes the fact that a cool place is not always the best place to store seeds.

The efficacy of below-freezing temperatures for storage has not been so generally known. A statistical analysis by Wakeley (21) of Barton's 1935 data on pine seed storage showed -4° C. significantly better than 5° C. for storage. Results of subsequent tests on these seeds and many others have confirmed and extended this conclusion, as has been demonstrated in the data already presented in this paper. A more concise presentation is to be found in Table VIII, a small portion of which has already been published (9). The striking differences in the two low temperatures for storage became evident only after extended periods of storage, i.e. longer than five years, and thus would be overlooked in experiments of short duration. Several reports in the literature refer to the keeping of seeds at temperatures below freezing, but most of them do not offer comparisons with those just above freezing. Beattie and Tatman (13) kept parsnip seed for six years at 20° or 40° F. Beattie and Boswell (12) found that onion seeds maintained their viability without significant loss for nine years. They recommended reducing the moisture content of these seeds to not over 6 per cent and storing in sealed containers held at 40° F. or lower. According to Brunson (14) most samples of seed corn stored at -20° C. for six years were practically as vigorous as when stored, and storage at 2° C. was nearly as good. Two to 4° C. has been recommended for all coniferous seed by Heit and Eliason (16). Noble fir seed can be held for five years at 15° F. (17), and a temperature of 32° to 39° F. is recommended for white pine seed storage (19). Storage effects on southern pine seeds have been summarized recently by Wakeley (22). Toole and Toole (20) found an advantage of -10° over 2° C. for storing soybean seed containing approximately 18 per cent moisture for six years. The viability of short-lived sugarcane seeds has been prolonged to two years by storage at 0° F. (15).

In the literature cited, the advantages of low temperature storage have certainly been recognized but, in most cases, the experiments did not continue long enough to show significant differences between above- and below-freezing temperatures. The data presented in this paper and the material included in the discussion have related to the longevity of seeds which tolerate drying. The effect of freezing on seeds with high moisture content is another matter and will not be considered here. New experiments have been started in this laboratory to determine the comparative effects of -4° C. and -18° C. on the keeping quality of vegetable seeds. In some cases a sufficient quantity of seeds has been stored to allow for 150 years of testing.

TABLE VIII
COMPARISON OF 5° C. AND -4° C. FOR MAINTAINING THE
VIABILITY OF SEEDS IN STORAGE

Seed	Moisture content, %	Per cent germination after storage for years							
		1		5		10		12-19	
		5° C.	-4° C.	5° C.	-4° C.	5° C.	-4° C.	5° C.	-4° C.
<i>Ulmus americana</i> 96*	Open	13	87	—	63	—	14	—	1 (12 yrs.)
	7	91	84	80	78	2	67	0	59 (12 yrs.)
	3	83	90	85	84	25	62	1	83 (12 yrs.)
	2	95	88	86	79	18	68	0	72 (12 yrs.)
<i>Pinus echinata</i> 59*	Open	64	43	49	72	14	64	3	36 (16 yrs.)
	Sealed	61	63	77	49	39	39	16	42 (16 yrs.)
<i>Lilium regale</i> 90*	Open	—	92	0	95	—	93	—	72 (15 yrs.)
	9.9	—	97	94	96	59	93	20	83 (15 yrs.)
	4.5	90	89	97	95	91	98	—	85 (15 yrs.)
<i>Gossypium</i> 67*	5	57	67	67	49	35	43	36	43 (13 yrs.)
	3	63	50	41	38	21	34	12	35 (13 yrs.)
<i>Gladiolus</i> 82*	Open	56	84	0	64	—	4	—	0 (12 yrs.)
	7.6	86	67	92	95	0	82	0	73 (12 yrs.)
	5.8	81	84	88	93	0	73	0	70 (12 yrs.)
<i>Delphinium</i> (annual) 57*	Open	28	44	7	17	—	6	—	— (19 yrs.)
	Sealed	59	60	50	61	35	48	—	54 (19 yrs.)
<i>Delphinium</i> (perennial) 43*	Open	3	44	0	37	—	6	—	— (19 yrs.)
	Sealed	39	42	33	42	5	50	—	41 (19 yrs.)
<i>Paeonia suffruticosa</i> 50*	Open	37	42	3	62	0	29	—	—
	Sealed	45	37	6	22	0	0	—	—
<i>Pinus taeda</i> 82*	Open	86	76	64	87	49	88	15	80 (12 yrs.)
	Sealed	89	74	68	62	41	69	13	73 (12 yrs.)

* Per cent germination at time of placing in storage.

Moisture content. The effect of moisture on the keeping quality of seeds is so closely related to temperature that it is very difficult to separate one from the other. A high moisture content is more deleterious at a high than at a low temperature as has been amply demonstrated by many workers. The harmful effect of a high moisture at a low temperature has not been reported so often, but has been definitely shown at a temperature of 5° C. by results from this laboratory.

The more rapid deterioration of seeds stored in sealed containers which are repeatedly opened and sealed as compared with seeds in sealed con-

tainers which are never opened from the time of storage to the time of testing as shown by data for dandelion, aster, *Verbena*, and *Gladiolus* above deserves some comment. Evidence in the form of delayed ill effects of opening and sealing by decreased initial moisture content of the seeds of dandelion leads to the conclusion that fluctuation in moisture content is harmful. Reference is made again to Figure 2 on this point. Moisture determinations made by drying the seeds in a vacuum at 75° C., have shown that seeds with 7.9 per cent moisture at the beginning gradually dry out to about 6 per cent with repeated opening of the storage can. As far as moisture content itself is concerned, this should be an advantage, but the fact is that dandelion seeds keep better with a constant moisture content of 7.9 than with a fluctuation downward. Deterioration of seeds with 3.9 per cent moisture is accelerated when repeated opening of the can has brought the moisture content up to about 6 per cent as shown by actual moisture determinations. Dandelion seeds with the intermediate initial moisture contents (6.2 and 5.0 per cent) were intermediate in their responses to fluctuation. It should not be assumed from this discussion that it is always better to seal seeds with high moisture than to leave them open. If the moisture content is excessive, there is increased injury in sealed containers, but within certain limits which probably vary for each kind of seed, fluctuation in moisture results in greater damage to the seed than could normally be expected from constant moisture content levels. This has been demonstrated experimentally for onion seeds (6).

Gas relations. The effect of a partial vacuum on longevity has been considered for several different forms, with varying results. Some benefits of a lessened oxygen supply may have been experienced by conifer seeds under certain storage conditions. On the other hand, aster and *Verbena* seeds were not improved by a partial vacuum under the conditions tried in this laboratory (5). It might be expected that when other storage conditions are unfavorable, there would be a favorable effect of reduced oxygen supply, but more work needs to be done to prove this point.

Testing technique. Many reports on seed viability do not include a description of the method used for germination tests. The importance of germination technique has been shown in this paper for soil tests of elm and pine. Obviously if improper germination methods are used they give an inaccurate picture of the germination capacity of a seed. A standardized procedure is very important, especially in tests in a greenhouse where temperature and light conditions vary greatly from day to day, and do not permit valid comparisons when tests are made from year to year. If controlled temperatures are available and suitable, they permit greater accuracy. In any case, the method used for testing viability should always be a matter for consideration and should be stated in a report.

Practical importance. It is very important to the seedsmen to be able to

keep surplus supplies for sale in later years. In some instances this has been done without the benefit of proper storage conditions for maintaining high quality seeds. As a result, everyone who buys seeds is apt to get lots which are of low quality or which fail to germinate. As more information is secured on the storage requirements, reputable seedsmen are building dehumidified or cold storage rooms. The possibility of holding seeds for at least two years reduces the amount of land needed for seed production since crops could then be alternated.

Many conifer trees do not set seed every year. There may be an interval of as much as 10 years between crops. This poses a serious problem in reforestation projects which depend on a constant supply of seeds for nursery use. Also direct reseeding of forest land could be seriously hampered by the lack of a seed supply in any given year. With the benefit of below-freezing storage, it is safe to hold conifer seeds for adequate periods. Such low temperatures automatically control insect damage, which is considerable in many seeds. Insect control has usually depended on the presence of certain insecticides which introduced the problem of injury to the seeds or to the persons handling the seeds.

The possibility of long-term storage is a boon to persons concerned with genetics or with plant introduction for it provides a constant source of valuable seed stock without the necessity of growing large numbers of plants and controlling seed production, a laborious and difficult process.

Granted that good storage conditions for a certain seed have been determined there are still questions which arise. Will the seeds from storage, especially low temperature storage, survive removal for packeting which must be done well in advance of the time the consumer buys and plants them? Furthermore, after packeting, seeds are sometimes stored under very unfavorable conditions on the retailers' shelves. Some experimental results on this problem in regard to onion seeds have been reported (10), demonstrating the efficacy of moisture-proof packets. The best storage condition determined for a specific seed, i.e. low moisture content and low temperature for seeds which tolerate drying, provides the seeds with maximum resistance to harmful conditions following packeting for sale. Additional work needs to be done in this field using different kinds of seeds, but the principles found for onion seeds will probably apply to many other species.

The type of plants which can be expected from old seeds is another matter of importance. It has been shown that *Verbena* plants from 9-year-old seeds and pepper and tomato plants from 13-year-old seeds of high germination capacity were of the same quality as plants from fresh seeds; plants from old aster seeds flowered earlier than those from fresh seeds; and lettuce seed stored for 13 years produced heads of larger weight than fresh lettuce seed. For details of these experiments the reader is referred

to a previous publication (11) which also contains a review of literature on the subject.

It is evident that the performance of any seed depends less upon its age than upon the conditions under which it has been stored.

SUMMARY

Effect of extended storage under various conditions on the viability of seeds of trees (*Abies*, *Pinus*, *Picea*, *Ulmus*); vegetables (*Daucus*, *Solanum*, *Lactuca*, *Allium*, *Capsicum*, *Lycopersicon*); dandelion (*Taraxacum*); flowers (*Callistephus*, *Verbena*, *Gladiolus*, *Delphinium*, *Paeonia*); and cotton (*Gossypium*) are reported and data given. Results showed the advantage of reduced moisture content and sealed containers; rapid deterioration at laboratory temperature and in a saturated atmosphere at 5° C.; retention of high germination capacity for approximately five years in sealed storage at 5° C., and for 10 years or longer at -4° C. The importance of an unbroken seal from the time of storage until the time of germination is emphasized since fluctuation of moisture content of the seeds caused by repeated opening and sealing of the storage container is harmful.

A discussion of some of the storage effects and their practical significance is given.

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INHERITANCE OF DDT TOLERANCE IN THE HOUSE FLY¹

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The introduction of DDT [2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane] in 1943 revolutionized house fly (*Musca domestica* L.) control. Wherever the material was used in conjunction with reasonable sanitary precautions, house flies were practically eliminated. After four to five years' use, however, less sensational results were obtained. This new development was soon correlated with the appearance of DDT-tolerant strains of house flies in such widely separated areas of the United States as California (14), New Jersey (2), and Illinois (4). Simultaneously, similar findings were reported in Italy (15, 20) and Sweden (23). Subsequently all strains no longer readily controlled with DDT, as well as with some of the more recently developed organic compounds, have been rather freely classed as resistant. The term tolerant is suggested as more correctly designating those strains of house flies with ability to withstand increased amounts of a toxicant.

Spontaneous development of tolerant strains in so many localities raised many questions concerning the fundamental nature of tolerance to insecticides and the possibilities of its being transmitted to future generations. Wiesmann (23) reported morphological differences between tolerant and susceptible strains collected at Arnaes, Sweden. It does not appear, however, that these differences could have given rise to such high levels of tolerance. Sternburg, Kearns, and Bruce (22) found that when DDT had entered the bodies of tolerant strains, it was rapidly metabolized into DDE [2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene] and into smaller amounts of DDA [bis-(*p*-chlorophenyl)acetic acid].

The resulting impact upon house fly control programs and upon tolerance to other chlorinated hydrocarbons, as well as other classes of insecticides, accentuated the seriousness of this development. A direct bearing was reflected upon the potential usefulness of different materials in alternating spray programs, in the development of tolerance in other insect pests, and in the outline of future research with organic chemicals.

The nearly universal use of DDT and appearance of subsequent tolerance poses aspects of scientific interest. Apart from the above economic ramifications, new ecological factors have been introduced into the in-

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sects' environment which would have a direct bearing upon any hypothesis of the phases of natural selection. Accordingly, in considering inheritance of DDT tolerance in house flies, it must be realized that the ability of any organism to adapt itself to a changing environment is in itself hereditary. The ability is transmitted to future generations. Whereas the ability to adapt is transmitted to future generations, an adaptation actually attained within a generation may not be hereditary and is not necessarily transmitted.

In the experiments to be reported below no difference in viability was noted between eggs of tolerant and susceptible strains temporarily immersed in solutions of DDT or its analogues. However, larval tolerance to residues of the same compounds appeared directly correlated with the degree of tolerance expressed by the adults. When pupae were treated, emergence and subsequent survival was inhibited in all strains, but markedly so in the laboratory strain. Hybridizing tolerant and susceptible strains attenuated the tolerance level. Backcrossing progeny of hybrid crosses to the more tolerant parent increased the level of filial tolerance. Conversely, backcrossing to the less tolerant parent decreased the filial tolerance level. Longer than normal life cycles, often associated with tolerant strains, appeared directly attributable to longer larval stages.

MATERIALS AND METHODS

HOUSE FLY STRAINS

Multi strain I, hereinafter designated as the Illinois strain, was reported (4) as exhibiting in excess of one thousand times the normal tolerance of house flies to residual deposits of DDT. It was the most tolerant strain used. The Bellflower strain (14) had demonstrated a field tolerance to DDT of approximately five hundred times the normal. It is referred to as the California strain. The Ellenville line of Barber and Schmitt (2) had displayed tolerance to DDT far in excess of the normal strain but the degree of tolerance has not been otherwise specified. Serving as the third comparative strain, it is herein designated as the New Jersey strain. A fourth strain in which tolerance to BHC (benzene hexachloride) had been developed (3) in consequence of accidental laboratory contamination, expressed a tolerance to DDT of approximately twice that of the laboratory strain, although it had never been exposed to DDT. It is referred to as the New Hampshire strain. A laboratory strain, devoid of any previous exposure to toxicants and with tolerance to DDT equivalent to that exhibited by house fly strains prior to development of DDT tolerance, was used as a control.

Rearing was carried out in accordance with specifications of the National Association of Insecticide and Disinfectant Manufacturers (17) for the Peet-Grady method. Colonies were maintained in wire mesh cages and

fed with evaporated whole milk diluted with three parts of water. Formalin (37 per cent formaldehyde) was added at 1:1500 as a preservative.

CHEMICALS

DDT and some of its analogues were used in studies of immature stages, with DDT only being considered in adult observations. Aerosol grades included DDT (containing 98 per cent of the *p,p'* isomer) and TDE [2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane]. Technical grade analogues included DFDT [2,2-bis(*p*-fluorophenyl)-1,1,1-trichloroethane], methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] containing 88 per cent methoxychlor and 12 per cent related compounds, and 2,2-bis(*p*-tolyl)-1,1,1-trichloroethane.

IMMATURE STAGES

Eggs. Randomized samples of eggs, not more than 24 hours old and representative of all egg masses deposited in the feeding dish by each representative strain, were removed and placed in 10 ml. of distilled water, gently agitated and allowed to settle. Those eggs and extraneous matter remaining afloat were discarded. Samples of test eggs were removed by a medicine dropper measuring 0.10 cm. inside diameter, allowed to settle to the orifice of the dropper, and then extruded into 10-ml. vials containing 5 ml. of a 10 per cent solution in acetone of each toxicant. A solution of 0.01 per cent rotenone in acetone (12) served as a standard. Controls consisted of acetone-treated and untreated eggs similarly processed.

All eggs were gently agitated in the respective vials and allowed to settle, a period of 15 to 30 sec. elapsing for any one immersion. Eggs were removed with a medicine dropper as above and extruded onto quadrants of scored filter paper in 9-cm. Petri dishes. These latter were left open for one hour to allow evaporation of the solvent. After drying, 1 ml. of water was distributed over the eggs to bring about a more even dispersal thereof and thus to facilitate subsequent differentiation between hatched and non-viable eggs. Because of the relative insolubility of the toxicants in water (rotenone 1:6,000,000) this procedure probably did not appreciably affect the actual deposit of toxicant upon the egg surface. Moisture content of the Petri dishes was maintained at a level sufficient to obviate excessive desiccation by placing a small piece of water-moistened cotton in the Petri dish apart from the eggs. Eggs were incubated at room temperature and all counts were made when 90 per cent hatching had taken place in the untreated controls.

Larvae. Tests with residues were conducted by adding 1 ml. of a 10 per cent solution in acetone of DDT or one of its analogues to a 9-cm. filter paper suspended on three needle points (correspondence with R. L. Metcalf, 1950). The solution spread evenly over the entire surface by capil-

larity, leaving a known amount of deposit evenly distributed. When completely dry, the filter paper was placed in a 9-cm. Petri dish (14). Food and moisture were provided by a small piece of absorbent cotton, moistened with diluted evaporated whole milk, placed in the center of the filter paper. Twenty-five two-day-old larvae were placed in the Petri dish and covered. Duplicate dishes were prepared for each strain and kept in a constant temperature and relative humidity chamber.

Pupae were removed daily as formed and placed in clean Petri dishes. Percentage ultimate survival was computed on the basis of the number of adults which emerged from the puparia and lived 48 hours or longer thereafter. Eight replicates at irregular intervals were used.

Pupae. Three-day-old pupae of uniform and representative size were selected from each of the strains. Their average weights in milligrams, computed from samples of 200 pupae from each strain, were Illinois 22, California 21, New Jersey 20, New Hampshire 17, and laboratory 24. Twenty-five pupae for each treatment were placed in a cylindrical No. 12 mesh wire container, approximately 1 cm. in diameter and 5 cm. in height, attached to a piece of nickel-chrome wire 20 cm. long. The upper end of the container was hinged on a wire loop for ease of opening. Pupae for any particular treatment were thus immersed for 15 to 20 sec. in appropriate tubes of 10 per cent solutions of the toxicants. Upon removal the pupae were gently shaken to remove any excess solution and emptied onto a 9-cm. filter paper in a Petri dish. After the solvent had evaporated, the filter paper was moistened with 1 ml. of water and covered. As adults emerged they were collected in tarlatan cloth cages (21), fed on diluted evaporated whole milk with which absorbent cotton had been moistened, and observed daily. Only those adults which survived for 48 hrs. or longer following emergence were considered to have successfully emerged. Eight replicates were conducted at irregular intervals, involving 400 pupae from each strain. Effectiveness of the compounds was computed in terms of average percentage of successful emergence.

ADULTS

Exposure. Adult flies were exposed to vaporized DDT evolved within an exposure chamber, by an electrically-heated, thermostatically controlled vaporizer, known commercially as the Aerovap.³ As herein employed the term aerosol, descriptive of vaporized DDT, is construed as including the entire mixture of air, liquid, and solid particles suspended therein, rather than the particles or parent substance thereof exclusive of the air. The Aerovap is composed of three cylindrical, open-top receptacles which fit one inside the other. The outer receptacle, constructed of bakelite, is attached by machine screws to an extension arm, for attachment to a wall

³ A product of American Aerovap Inc., 170 West 74th St., New York 17, N. Y.

surface. The base of the outer receptacle is equipped with female electrical contacts which receive the male elements of and serve to mount the intermediate cast aluminum receptacle. The intermediate receptacle is encompassed by the heating element, supporting and adjusted by a thermostatic control, adapted for external adjustment. The inner receptacle is an aluminum insecticide cup (improved glass cups are now in use) measuring approximately 8 cm. inside diameter and 5.5 cm. deep, with a holding capacity of about 150 g. of DDT. It fits snugly into the intermediate receptacle.

The exposure chamber consisted of a 500-cu. ft. cube-shaped plywood enclosure, with exterior studding and smooth interior. Ventilation was effected before and after each exposure by a motor-driven exhaust fan at the upper right front corner. The fresh air inlet was at the lower left rear, both inlet and outlet ports being closed during exposures by sliding metal doors. Rectangular machine-sewn tarlatan cloth cages (21) measuring $6.5 \times 6.5 \times 14$ inches were used for exposure of adults to the aerosol.

Feeding cups and glass jars containing puparia were removed from the cages prior to exposure. In order to eliminate the probability of contamination any cage containing more than two dead flies prior to exposure was discarded. Only flies three to five days old were used, flies of the same age being used throughout any test. Cages were arranged in Latin square design, placed on clean newspapers. Following exposure, cages were removed to an uncontaminated area and food provided. Mortality was computed (1) 24 hours after initiation of exposure.

LD₉₀—laboratory strain. Flies were exposed for various periods of time, ranging from thirty minutes to five hours, to determine a length of exposure which would effect an approximate LD₉₀ in the laboratory strain and simultaneously a measurable degree of mortality in the presumed most tolerant strain, i.e., the Illinois strain. Weights of DDT evolved for each exposure period were determined and compared with the respective mortalities produced.

Paired and mass crosses. Individual paired crosses and their reciprocals were carried out between the laboratory and Illinois strains. Mass crosses and reciprocals, consisting of 20 males and 20 females from each of these strains, were simultaneously effected. Progeny from both paired and mass crosses were reared through ten successive filial generations, each generation being reared apart from its respective parental generation. Representative samples of each generation, together with samples from the laboratory and Illinois strains, were exposed to aerosol phase DDT as previously described.

Crosses between tolerant and laboratory strains. Mass crosses and their reciprocals were carried out, as above described, between the laboratory strain and the California, New Jersey, and New Hampshire strains. Progeny from each cross and its reciprocal were reared through ten generations,

each generation being reared apart from its respective parental generation. Representative samples of each generation, together with samples from each parental strain, were exposed as above.

Inter-tolerant strain crosses. The same procedure as above was used in crosses between tolerant strains. Since it was not possible to effect all crosses and subsequent exposures at the same time, percentage mortalities of the parental strains are averages of the several tests in which these strains were hybridized and their progeny exposed.

BACKCROSSES

Sufficient of the F_1 generations from Illinois \times laboratory and laboratory \times Illinois were retained to propagate the progeny as separate inbred cultures, each generation being reared apart from its respective parental generation. The remaining progeny was divided into five categories, for processing as follows: (A) exposure to DDT for tolerance level determinations; (B) males backcrossed to females of parental Illinois strain; (C) females backcrossed to males of parental Illinois strain; (D) males backcrossed to females of parental laboratory strain; (E) females backcrossed to males of parental laboratory strain. The degree of tolerance expressed by each of the foregoing categories was computed from mortalities induced by four-hour exposures to DDT in continuous aerosol phase. Observations were extended through ten filial generations. In view of the differences in tolerance expressed by the F_1 generations of Illinois \times laboratory and laboratory \times Illinois, backcrosses were initiated from progeny of both the cross and the reciprocal cross and consequently from two different levels of tolerance.

VARIATION IN LIFE CYCLES

During the early part of this study it became apparent that the length of the life cycles of the different tolerant strains differed from each other and from the laboratory strain. Although reared under uniform conditions these differences had apparently persisted over a period of three months prior to the subject observations.

Randomized samples of eggs, all less than 12 hours old, were taken from each of the strains and reared in the manner previously described. Forty-eight hours after the eggs were seeded in the rearing medium, the sand surrounding each of the jars was removed, covered, placed in a constant temperature and relative humidity chamber, and replaced with fresh sand. This removal and replacement of sand was repeated every 12 hours for 9 days. All larvae that crawled out of the rearing jar, preparatory to pupation, during any 12-hour period, were thus grouped together. Three days after removal of the sand, the larvae had completed pupation and were screened therefrom, counts being made of the numbers in each group.

Eight such observations were made during a period of three months. Since wide variation was encountered in the number of eggs selected from each strain, the average number of pupations effected during any 12-hour period is expressed in percentage, as the portion of the total pupation which occurred during that particular 12-hour period.

Approximately eight months following the above observations, a second series of observations was initiated, prompted by a twofold objective. The length of the larval stages in the New Jersey and California strains, although apparently still at some variance with that of the laboratory strain, appeared to more nearly coincide with the laboratory strain than they had eight months previously. Secondly, the relative tolerance to DDT of these two strains, i.e., the New Jersey and California strains, appeared somewhat lower than in previous determinations. A series of observations, replicated eight times over a three-month period as above, was carried out.

RESULTS AND DISCUSSION

IMMATURE STAGES

Eggs. In 1949 treatment of eggs of the California, New Hampshire, and laboratory strains with DDT or its analogues did not affect their viability. In 1950 a second series of tests, including, in addition to the foregoing, eggs of the Illinois and New Jersey strains, acquired during the interim, showed no difference in percentage hatching of eggs of the different controls and those eggs treated with DDT or its analogues. Rotenone (0.10 per cent solution in acetone) induced mortality in approximate agreement with the results of other workers (12, 19).

Larvae. Various opinions have been expressed concerning toxicity of DDT to house fly larvae. It appears (4, 6) that conditioning the larval stage to this toxicant fosters a more rapid increase in tolerance in the adult. In field practice the use of DDT for house fly control in manure piles and other breeding places actually parallels this artificially-induced laboratory practice. The foregoing prompted a twofold objective in larval observations, (A) to determine the relative larvicidal effectiveness as residual deposits of DDT and its analogues against the tolerant and laboratory strains, and (B) to ascertain whether the presence of DDT tolerance in the adult house fly in any manner affects tolerance to DDT of the larval stage. The relative larvicidal effectiveness of residues of DDT or its analogues (Table I) shows DFDT to be the most toxic, average survival of all strains exposed thereto being 52 per cent. In contrast DDT indicates the least larvicidal effectiveness, average survival therefrom being 65 per cent. TDE, methoxychlor, and 2,2-bis(*p*-tolyl)-1,1,1-trichloroethane are all intermediate in effectiveness and not significantly different from one another, the respective survival averages of all strains treated being approxi-

mately 59 per cent. The laboratory strain expressed little variation in tolerance to the different chlorinated compounds.

Pupae. Effectiveness of DDT or its analogues as control measures for the pupal stage of the house fly is not of particular economic importance. Various investigators have reported that use of DDT residues on house fly puparia has inhibited successful emergence of adults to such an extent that only a few actually emerged and of these a comparatively small number lived more than a few hours. Observations of comparative tolerance

TABLE I
PERCENTAGE SURVIVAL OF 400 LARVAE OF EACH STRAIN EXPOSED TO
SURFACES TREATED WITH DDT OR SOME OF ITS ANALOGUES

Toxicant	Per cent survival of various strains					
	Ill.	Calif.	N. J.	N. H.	Lab.	Mean
DDT	86	75	65	52	47	65.0
DFDT	68	55	50	44	41	51.7
TDE	81	65	57	50	42	59.0
Methoxychlor	87	64	52	45	44	58.3
2,2-Bis(<i>p</i> -tolyl)- 1,1,1-trichloroethane	74	65	56	50	50	59.0
Acetone	95	93	92	94	93	
Water	95	94	94	94	94	
Mean of DDT and analogues	79	65	56	48	45	
LSD 1:19 Single determination					12	
Mean of 5 determinations					5.3	

of the pupal stage of various strains to the subject toxicants have been included to ascertain the presence or absence of correlation with tolerance in adults.

March and Metcalf (14) reported that enormous differences in susceptibility of various house fly strains to applications of DDT were not due to variations in the weights and vigor of the flies. Comparison of percentage emergence and survival (Table II) from pupae treated with DDT or its analogues with average pupal weights indicates that apparently no correlation exists between pupal weight and susceptibility of the emerging adult to applications of DDT or its analogues.

Marked toxicity by DDT and its analogues to the pupal stage of all strains is apparent. No appreciable difference is noted, however, in response of the various tolerant strains to any particular treatment, all showing a higher percentage of emergence and consequently a higher level of tolerance to these compounds than the laboratory strain. A comparison of the relative toxicity of the various compounds places DFDT significantly apart, all tolerant strains being more susceptible to this material. The laboratory strain shows a marked susceptibility to DFDT, far in excess of

TABLE II
PERCENTAGE ADULT SURVIVAL FROM 400 PUPAE OF EACH STRAIN
TREATED WITH DDT OR SOME OF ITS ANALOGUES

Toxicant	Per cent survival of various strains					
	Ill.	Calif.	N. J.	N. H.	Lab.	Mean
DDT	64	65	63	65	51	61.6
DFDT	52	51	51	53	20	45.4
TDE	69	66	67	67	52	64.2
Methoxychlor	61	62	62	64	55	60.8
2,2-Bis(<i>p</i> -tolyl)- 1,1,1-trichloroethane	62	59	58	60	43	56.4
Acetone	92	91	93	92	92	
Untreated	97	97	96	97	95	
Mean of DDT and analogues	62	61	60	62	44	
LSD 1:19 Single determination					12.5	
Mean of 5 determinations					5.6	

that to any other compound. Percentage emergence from pupae of the New Hampshire strain is comparable to that of other strains, irrespective of the treatment rendered.

ADULTS

Mortality versus weight of DDT evolved. The average amount of DDT evolved during different exposure periods increased at an approximate rate of 0.02 g. per 30 minutes of increased exposure. Percentage mortality produced by 30 minutes' exposure was negligible, since there was less than 0.005 g. of toxicant evolved. Since approximately 20 minutes are required to allow the heating unit of the Aerovap to produce a well temperature suitable for vaporization of DDT ($130^{\circ} \pm 1^{\circ} \text{C.}$), the remaining 10 minutes are not sufficient to produce an appreciably toxic aerosol concentration. Respective weights of DDT dispersed tended to coincide with increased mortality, approaching the pattern of a normal mortality-concentration curve. Average mortalities produced at the 0.19-g. dispersal level were approximately 90 per cent for the laboratory strain and 8 per cent for the Illinois strain. Intermediate levels of tolerance, represented by intermediate levels of mortality, decreasing in the order named, were reflected in the California, New Jersey, and New Hampshire strains. Approximately four hours were required to evolve 0.19 g. of DDT. Five exposures were conducted, using 5,000 flies per exposure.

Mortality versus length of exposure period. Mortality produced at the 30-minute and one-hour exposure levels was too low to allow clear differentiation between strains, the laboratory strain displaying an average mortality of 17 and 36 per cent respectively. No tolerant strain showed mortality at the 30-minute level and all were below 10 per cent at the one-hour

level. Because of this low mortality and accompanying uncertainty of clear differentiation between mortalities of the different strains, these data are omitted from Figure 1.

Percentage mortality produced increases as the length of the exposure period increases. At the four-hour exposure level, the highest mortality, that of the susceptible laboratory strain, was approximately 90 per cent while the lowest mortality, that of the Illinois strain, was 10 per cent. In-

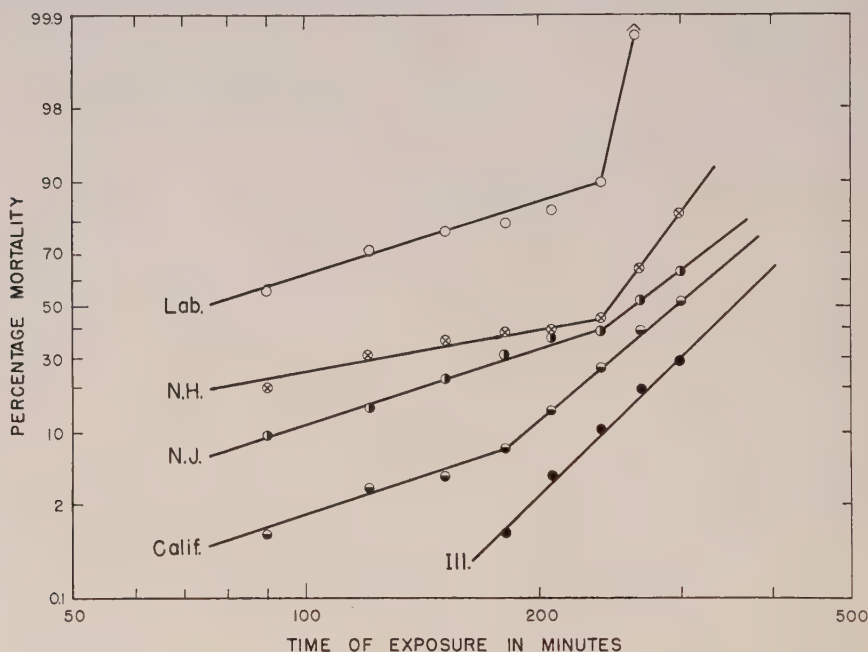


FIGURE 1. Dosage-response curves for the toxicity of vaporized DDT to various strains of house flies.

intermediate levels of mortality, representing the remaining strains, were California 26 per cent, New Jersey 39 per cent, and New Hampshire 45 per cent. At the LD₅₀ level, the following relative order of tolerances were expressed: Illinois 4.5, California 3.7, New Jersey 3.3, New Hampshire 3.0, and laboratory 1. Weights of DDT evolved during the four-hour exposure periods varied from 0.158 g. to 0.237 g., with a mean average dispersal weight of 0.194 g. Ten exposures were conducted, using 10,000 flies per exposure.

Direct comparison herewith of the tolerance levels reported (2, 3, 4, 14) for the subject strains is obviated because of the wide divergence in the respective techniques employed. When DDT is evolved in continuous aerosol phase two physical states of the compound result. The air becomes satu-

rated with the toxicant in vapor phase, and simultaneously supports particles of the compound derived from condensation of that portion of the vapor phase in excess of the saturation requirements. Bruce (4) made topical applications of the toxicant to the thorax, whereas March and Metcalf (14) employed residual deposits. Blickle, Capelle and Morse (3) used a modification of the insect toximeter (16) whereby a known volume of spray under constant pressure was directed from an artist's air brush toward a rotating cage of flies.

Paired and mass crosses. No difference in tolerance to DDT could be detected (Table III) between progeny of the paired crosses and the mass crosses. The degree of tolerance displayed by the Illinois strain is diluted in the progeny when that strain is crossed with the laboratory strain. A constant difference in level of tolerance to DDT was observed between the

TABLE III
PERCENTAGE MORTALITY IN PROGENY OF CROSSES BETWEEN
ILLINOIS AND LABORATORY STRAINS

Progenitor	Percentage mortality in successive filial generations									
	1	2	3	4	5	6	7	8	9	10
Illinois (a)	7	5	4	8	6	12	9	8	10	7
Ill. × lab. (paired) (b)	38	40	36	37	39	41	43	40	38	43
Ill. × lab. (mass) (c)	40	36	37	41	42	38	40	42	36	37
Lab. × Ill. (paired) (b)	21	19	13	13	16	19	22	18	23	19
Lab. × Ill. (mass) (c)	18	23	21	15	19	14	19	21	18	21
Laboratory (a)	94	91	85	91	89	96	93	90	87	92

(a) 1200 flies used in each of two replicated exposures per generation.

(b) 20 individual paired crossings, replicated 5 times per generation.

(c) 20 males and 20 females in each of 20 mass crossings per generation.

progeny of the cross and the reciprocal cross. In each instance the filial tolerance displayed by progeny of laboratory × Illinois exceeded that of the reciprocal cross. Although this difference remained relatively constant throughout ten successive generations, it is questionable whether such difference would indicate sex-linked characteristics. In consequence of similar crosses Bruce (4, 5) concluded that the resistance factors were carried by both males and females of the tolerant strains.

Crosses between tolerant and laboratory strains. In each cross between a tolerant strain and the laboratory strain, the degree of tolerance expressed by the successive filial generations (Table IV) was a dilution of that expressed by the tolerant parental strain. This level was about midway between that expressed by the respective parental strains. No appreciable increase or decline in tolerance was noted in the progeny of either cross during ten ensuing generations.

The extent to which the manner of origin might influence tolerance expression within filial generations is, in the absence of supporting data, a

matter of conjecture. The difference in degree of tolerance expressed by the progeny of crosses and reciprocal crosses between the laboratory strain and the Illinois (Table III) or California (Table IV) strains would indicate that the influence of some factor (or factors) is being expressed. Crosses between two strains possessing tolerance to a common toxicant might conceivably nullify the degree of tolerance presumably contributed by each strain and thus give rise to progeny devoid of tolerance to that particular toxicant. Contrariwise, progeny could exhibit an increase in tolerance in excess of that previously expressed by either parental strain. In view of the

TABLE IV
PERCENTAGE MORTALITY IN PROGENY OF CROSSES BETWEEN
LABORATORY AND TOLERANT STRAINS

Progenitor	Percentage mortality in successive filial generations									
	1	2	3	4	5	6	7	8	9	10
Laboratory	90	92	92	92	90	92	93	91	89	95
Lab. × Calif.	43	42	40	42	43	43	45	43	46	44
Calif. × lab.	53	54	55	54	56	53	55	57	51	62
California	15	16	16	13	10	14	15	12	18	16
Lab. × N. J.	52	52	53	49	53	52	50	56	48	61
N. J. × lab.	53	54	53	56	51	53	58	49	55	48
New Jersey	26	30	29	32	26	28	30	24	27	28
Lab. × N. H.	70	74	66	69	70	67	69	70	76	68
N. H. × lab.	72	70	68	64	74	65	75	68	73	74
New Hampshire	45	52	43	41	58	51	62	43	41	45

work of Hough (8, 9, 10, 11), Dickson (7), and others, however, it would be more logical to anticipate a dilution of the tolerance displayed by the more tolerant strain and a simultaneous increase over the tolerance inherent in the less tolerant strain. The resultant composite level would thus evolve into an expression of tolerance approximately midway between those of the parental strains.

Although conditioned to BHC and apparently having concurrently developed a lesser tolerance to DDT, the New Hampshire strain appears capable of transmitting this DDT tolerance in greater or lesser amounts to successive generations, whether crossed with a DDT-tolerant strain or with a susceptible laboratory strain.

Inter-tolerant strain crosses. Consideration of the data presented in Table V indicates a rather uniform pattern of tolerance transmission, whether evolving from a cross between two DDT-tolerant strains or from a cross between a DDT-tolerant strain and a BHC-tolerant strain, the latter with secondary tolerance to DDT. All progeny express a tolerance to DDT somewhat below a point midway between the expressed levels of the parental strains. The degree of tolerance assumed by the F_1 generation appears to become well established within that generation and to be ca-

pable of expression at a constant equivalent level throughout ten successive filial generations.

The same differences in tolerance levels evolving from crosses and reciprocal crosses previously noted (Table IV) are expressed between crosses

TABLE V
PERCENTAGE MORTALITY IN PROGENY OF CROSSES BETWEEN
TOLERANT STRAINS

Progenitor	Percentage mortality in successive filial generations									
	1	2	3	4	5	6	7	8	9	10
Illinois	8	6	4	8	8	8	9	9	7	6
California	15	14	12	16	13	17	14	14	16	12
New Jersey	29	27	31	27	32	26	30	28	25	31
New Hampshire	45	48	40	48	49	43	40	49	51	39
Ill. × Calif.	14	12	8	14	11	12	16	12	7	12
Calif. × Ill.	10	8	10	13	10	10	16	9	18	7
Ill. × N. J.	22	22	23	23	25	19	19	24	21	24
N. J. × Ill.	18	17	18	21	21	16	16	20	14	17
Ill. × N. H.	28	28	27	28	29	25	30	30	24	26
N. H. × Ill.	18	17	18	19	23	23	13	14	22	18
Calif. × N. J.	20	18	16	21	21	16	21	18	15	20
N. J. × Calif.	19	15	20	16	21	21	15	18	17	18
Calif. × N. H.	26	22	27	27	23	29	29	25	22	28
N. H. × Calif.	22	28	23	26	27	24	20	20	28	27
N. J. × N. H.	29	31	34	33	29	36	33	28	38	31
N. H. × N. J.	33	32	31	38	37	30	30	36	31	31

and reciprocal crosses of the Illinois or California strains with other tolerant strains. Again a similar difference was not noted between crosses and reciprocal crosses of the New Jersey and New Hampshire strains.

BACKCROSSES

Progeny of Illinois × Laboratory

Tolerance of inbred filial culture. The tolerance of ten successive filial generations, propagated as an inbred filial culture, followed the same general pattern displayed by progeny of a similar cross discussed earlier (Table III), and continued rather constantly at this level.

Filial males backcrossed to parental Illinois females. The offspring of this cross and similarly of subsequent equivalent crosses were divided into two groups, one group being exposed to the vaporized DDT while the other group was backcrossed to parental Illinois females. Exposure of a portion of the progeny of each cross and backcrossing the other portion with parental Illinois females was continued throughout ten generations. The degree of tolerance reflected (Table VI) by the progeny of these backcrosses indicated a progressive but gradual increase in tolerance with each successive backcross.

Filial females backcrossed to parental Illinois males. Following the same

procedure, a portion of the F_1 females was backcrossed to males of the parental Illinois strain. Successively a portion of the progeny of this cross and of subsequent equivalent backcrosses was exposed to the toxicant while the respective remaining portions were backcrossed to males of the parental Illinois strain. Tolerance was slightly higher (Table VI) than expressed by progeny of equivalent backcrosses to the parental Illinois females. Whereas the same progressive increase in tolerance was noted in each successive backcross, a higher level was induced initially and continued throughout.

TABLE VI
PERCENTAGE MORTALITY IN PROGENY OF SUCCESSIVE BACKCROSSES

Progenitor	Percentage mortality in successive filial generations									
	1	2	3	4	5	6	7	8	9	10
Illinois	7	8	10	8	6	7	11	11	8	11
Filial females backcrossed to Illinois males	38	33	29	25	22	20	19	19	17	16
Filial males backcrossed to Illinois females	38	35	32	29	26	25	24	23	21	21
Illinois \times lab.	38	36	38	40	36	35	37	40	36	40
Filial females backcrossed to laboratory males	38	46	60	63	63	67	73	75	77	78
Filial males backcrossed to laboratory females	38	51	57	61	64	68	71	73	75	79
Laboratory	91	88	89	90	89	91	92	94	92	95

Filial males backcrossed to parental laboratory females. With each successive backcross of filial males to parental laboratory females, the level of tolerance was progressively reduced (Table VI), the rate of reduction appearing somewhat more abrupt in earlier generations and becoming more gradual in succeeding generations.

Filial females backcrossed to parental laboratory males. Tolerance expressed (Table VI) by the respective progeny was nearly identical to that of the reciprocal backcrosses, i.e., between filial males and parental laboratory females. Although variation is to be noted between the two lines of tolerance as expressed by any one generation, the actual average expressed throughout ten generations is about equivalent.

The patterns of tolerance evolving from the foregoing are nearly symmetrically divergent. With each successive backcross to the tolerant parental strain, the level of tolerance is progressively increased. With each successive backcross to the susceptible parental strain, the level of tolerance is progressively reduced. Filial tolerance levels in both increasing and de-

creasing patterns are below the midpoint for the tolerance of the respective patterns, being much lower in progeny of backcrosses to the tolerant than to the susceptible parent.

Progeny of Laboratory × Illinois

In contrast to the foregoing backcrosses, the F_1 generation of laboratory × Illinois was herein used as the starting point. The same procedure was used as in the preceding backcrosses, with the same sequence of exposures and backcrosses to tolerant and susceptible parental strains (Table VII).

TABLE VII
PERCENTAGE MORTALITY IN PROGENY OF SUCCESSIVE BACKCROSSES

Progenitor	Percentage mortality in successive filial generations									
	1	2	3	4	5	6	7	8	9	10
Illinois	7	8	10	8	6	7	11	11	8	11
Filial females backcrossed to Illinois males	38	33	29	25	22	20	19	19	18	16
Filial males backcrossed to Illinois females	38	34	31	29	26	25	24	23	23	22
Lab. × Illinois	38	36	38	40	36	35	37	40	37	40
Filial females backcrossed to laboratory males	38	46	60	63	63	67	73	75	78	79
Filial males backcrossed to laboratory females	38	51	57	61	65	68	71	73	77	81
Laboratory	91	88	89	90	89	91	92	94	92	95

Tolerance of inbred filial culture. Ten generations of inbred progeny displayed about the same tolerance indicated by earlier equivalent crosses.

Filial males backcrossed to parental Illinois females. This pattern of tolerance resembled that displayed by progeny of the backcross between parental Illinois females and filial males. Whereas the actual level of tolerance in the present progeny is somewhat higher than indicated by progeny of an equivalent cross in the foregoing (Table VI), this is attributed to the higher level of tolerance inherent within the males serving as parental males in the present backcross. A gradual increase in tolerance is noted.

Filial females backcrossed to parental Illinois males. Tolerance was somewhat higher than indicated by progeny of the reciprocal backcross, the same general pattern of increasing tolerance previously noted being observed. The initial level of tolerance was higher than in progeny of the reciprocal backcross and continued so throughout.

Filial males backcrossed to parental laboratory females. Apart from initiat-

ing at a higher level of tolerance in consequence of higher parental tolerance, the same pattern of tolerance was expressed as by progeny of the backcross between the first F_1 group and the parental laboratory females. Rates of progressive loss in both instances are about equivalent.

Filial females backcrossed to parental laboratory males. Tolerance was about the same as observed in progeny of the preceding backcross between filial males and laboratory females.

Recognizing attenuation of tolerance through interbreeding with susceptible or less tolerant strains, as well as the gradual increase or decrease in tolerance induced by successive backcrosses, it is suggested that tolerance in the house fly may be a composite or summation expression of several factors. According to Mendelian laws of segregation, in a hybrid homologous genes of an allelic pair at a given locus segregate from one another during meiosis. As a result only one member of a pair of genes is present in each gamete. The genetic constitution of any organism therefore is the result of the particular gametes which unite when that organism is formed. Since the degree of expression of some characters may be clearly defined qualitatively, this expression is probably dependent upon one or a very few sets of alleles. Such variations are usually discontinuous. Other characters may be expressed quantitatively and are generally continuous. Characters which vary continuously are generally dependent upon many sets of alleles which express no particular dominance and are probably, therefore, cumulative in their influence. According to the multiple factor theory, several sets of alleles may produce equal and cumulative effects on an individual character.

In view of the apparent decline in tolerance in the California, New Jersey, and New Hampshire strains, and apparent lack thereof in the Illinois strain, two possible aspects of the tolerance complex may be considered. The first aspect, including the New Jersey, New Hampshire, and California strains, loses its tolerance when deprived of exposure to the conditioning toxicant. The California strain maintained its tolerance, without exposure to DDT, for 15 generations prior to rearing at this laboratory, and through an additional 16 generations subsequent thereto. After 34 generations of rearing at this laboratory, however, a decline in tolerance was noted. The New Jersey and New Hampshire strains, displaying relatively low initial tolerance to DDT, had not become extensively adapted to this toxicant. In the absence of exposure, a regressive but gradual loss of expression has taken place in the factors governing tolerance expression. This regression may likewise be applied to the California strain. In this instance, however, the higher initial level of tolerance would indicate that a more extensive degree of adaptation had taken place. Two phases of tolerance must be considered in application of the regression concept to the California strain. A marked decline in tolerance has taken

place. Secondly, a considerable degree of tolerance remains. The original level of tolerance probably was the aggregate expression of the cumulative effect of several factors governing the expression of tolerance. If a portion of these factors, because of lack of exposure to the toxicant, gradually lose their adaptiveness to the compound, an attendant loss in tolerance ensues. The degree of tolerance remaining is ascribed to those factors which, having become adapted to the toxicant, concurrent with the original expression of tolerance, have not as yet lost this adaptation, despite lack of exposure to DDT.

Upon this premise then, the original level of tolerance would be the aggregate expression of the cumulative effect of those factors which had become adapted to DDT as a part of their environment. Since several factors must have become adapted in the original expression of tolerance, and since only a portion of this tolerance has been lost, the degree of tolerance now remaining probably is the cumulative effect of those adapted factors, as yet non-reverted, which express themselves collectively.

No sharp line may be drawn demarking the California strain from the second aspect above indicated. Whereas the foregoing strains each represent a type in which the original tolerance has been gradually reduced because of relatively unstable adaptation to the toxicant concerned, the second aspect, exemplified by the Illinois strain, has indicated no decline in tolerance. Originally obtained as a highly purified strain with a high degree of tolerance to DDT, this strain may be construed as representing a tolerance complex in which a high degree of adaptation had been effected.

This tolerance had been progressively increased generation by generation. Attendant therewith, a step by step progressive adaptation had been effected. When the peak of tolerance was expressed and the most extensive degree of adaptation had been accomplished, a multi-factor complex had become established as the degree of tolerance expressed. Since there are numerous genes involved in a multi-factor complex as here proposed, a reversion process concomitant to loss of tolerance would be accomplished gradually through regression to the normal status of the factor concerned. Since the Illinois strain has been maintained as a pure inbred line, this process of regression probably would proceed slowly. On the basis of observations herein noted, no appreciable degree of loss of tolerance and presumably no appreciable degree of regression has yet occurred in this strain.

Tolerance patterns expressed in consequence of successive backcrosses (Tables VI and VII) indicate similarity in behavior of the tolerance complex, whether backcrossing to the tolerant or susceptible parent. Loss of tolerance by interbreeding with susceptible flies is conjoined with a concomitant increase in tolerance in consequence of interbreeding with

tolerant flies, the rate of increase or loss being progressively modified by each successive generation of interbreeding. It seems probable that the factors governing tolerance expression are present in both tolerant and susceptible strains. Because of certain ecological aspects to which the now tolerant strain had at some time previously been exposed, this strain is now capable of tolerance expression. Contrariwise, the susceptible strain, devoid of these particular ecological exposures, has not had opportunity to become adapted to these ecological variants and, therefore, is not now capable of tolerance expression. It is very probable that there are flies, or strains of flies which, regardless of the stimuli to which exposed, would never become adapted to tolerance expression.

Whether the conditioning toxicant is the sole contributing factor in establishment of this ecological niche, favorable for adaptation to the toxicant, is doubtful. Since the BHC-tolerant strain from New Hampshire was presumably conditioned by and to BHC, it nevertheless developed a lesser tolerance to DDT. In several instances the expression of tolerance to a particular toxicant has reportedly been accompanied by a simultaneous lesser adaptation to certain other toxicants. It would seem that any toxicant is capable of inducing a progressive cumulative expression of factors which, having quantitatively attained equilibrium with the ecological stimuli in the niche to which exposed, are able to adapt themselves progressively and eventually express themselves as a composite or aggregate complex, resulting in increased tolerance and eventual intensified tolerance to that particular toxicant. Greater resistance to hydrocyanic acid gas was observed (18) in red scale on trees fumigated regularly. Greater tolerance to DDT has been observed where this compound has been repeatedly applied.

VARIATION IN LIFE CYCLES

The peak of pupation in the laboratory strain precedes (Table VIII) that of the Illinois strain by approximately 24 hours, while the peaks of the New Jersey and California strains are generally midway between those of the laboratory and Illinois strains. The peak of the New Hampshire strain

TABLE VIII
PERCENTAGE PUPATION PER TWELVE-HOUR PERIOD

Strain	Per cent pupation of various larvae of different ages in days									
	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8
Illinois	0	2	4	9	27	30	20	5	2	1
California	3	4	7	26	31	16	8	3	2	0
New Jersey	4	6	9	23	28	14	8	5	3	0
New Hampshire	7	12	29	23	13	8	5	3	0	0
Laboratory	5	12	25	32	15	5	3	2	1	0

precedes that of the laboratory strain by about 12 hours and that of the Illinois strain by about 36 hours. The rate of pupation in the New Hampshire strain rises rapidly to its peak and then declines gradually. Rates of rise and fall in pupation curves of the other strains are quite uniform.

During a second series of observations, eight months subsequent to the preceding, it was noted that the larval stage of development in both the Illinois and laboratory strains remained relatively constant, when compared to earlier observations (Table VIII). The peak of the New Hampshire pupation shifted slightly toward an increase in length of larval stage. The greatest change, however, was in the comparatively marked reduction in length of larval stage in both the New Jersey and California strains, the peak of pupation in both strains occurring about 12 hours earlier than previously and coinciding approximately with that of the laboratory strain.

In view of the above apparent shortening of life cycles in the New Jersey and California strains, the concomitant increase in length in the New Hampshire strain, and the suspected lower level of tolerance above indicated, a second relative tolerance level determination was carried out. The average percentage mortality of 15 exposures for the various strains was Illinois 6, California 28, New Jersey 56, New Hampshire 66, and laboratory 92.

It is apparent that tolerance of the Illinois strain has remained relatively constant, whereas mortality in the laboratory strain is equivalent to earlier findings. Mortality in the New Jersey and California strains has increased markedly, indicating an approximate doubling of the former respective susceptibility levels. The New Hampshire strain has declined in tolerance but not so markedly as the New Jersey and California strains.

A matter of considerable current interest in the problem of resistance in house flies is the apparent longer life cycle (13) conjoined with tolerant strains. Two contrasting lines of reasoning may be considered on the basis of the foregoing data. Decrease in length of larval stage of development in the New Jersey and California strains has been accompanied by reduction in tolerance to DDT. However, an increase in length of larval cycle in the New Hampshire strain has likewise been accompanied by a decline in tolerance. Tolerance may apparently be accompanied by longer than normal life cycles but since the converse is apparently also true, it is suggested that longer than normal life cycles are not of necessity associated with tolerance to insecticides.

A further interpretation of differences in length of larval stage and consequently of life cycles evolves from consideration of environment and its ecology. Despite uniform rearing for all strains, propagated as inbred cultures and, therefore, devoid as far as known of the introduction of any element of digression from this uniformity, a gradual shift in length of life

cycles has nevertheless been noted. Such a shift would indicate that the rearing environment was not optimum and that preference for a life cycle other than being experienced was being expressed by the strains concerned. It may be concluded that a longer than normal life cycle, when associated with tolerance to insecticides, is actually the response of the individual strain to the environmental conditions to which subjected and that association with tolerance is only coincidental.

Whereas tolerance and length of life cycle have herein been dissociated from any interdependence, it is recognized that certain environmental stimuli may become conjointly resolved into an ecological niche favoring a longer life cycle and retention of the tolerance level then being expressed. Such a combination, however, is produced by expression of ecological factors upon two dissociated phases which, as a result, appear interrelated.

SUMMARY

Relative tolerance to DDT, TDE, DFDT, methoxychlor, and 2,2-bis(*p*-tolyl)-1,1,1-trichloroethane was compared in the immature life stages of the Multi strain I (Illinois), Bellflower (California), and Ellenville (New Jersey) DDT-tolerant strains, a BHC-tolerant New Hampshire strain, and a non-tolerant laboratory strain of house flies (*Musca domestica* L.). No effect upon hatching of eggs of tolerant or susceptible strains was induced by temporary immersion in 10 per cent acetone solutions of the toxicants. Relative tolerance of larvae of tolerant strains to residual deposits of DDT was directly correlated to the degree of relative tolerance to continuous phase aerosol DDT expressed by the adults. DFDT was the most toxic and DDT the least so, remaining analogues appearing intermediate in effectiveness. Emergence from puparia was markedly inhibited by treatment with DDT or its analogues. However, all tolerant strains, including the BHC-tolerant strain, exhibited a higher percentage emergence than the laboratory strain. In contrast to its apparent larvicidal effectiveness, DFDT was significantly less toxic than other compounds to the pupal stage. No significant difference was observed in the response of the different tolerant strains to treatment with the remaining toxicants. There was no apparent correlation between pupal weight and susceptibility of the emerging adult to the various treatments.

Exposure of adults for four hours to continuous phase aerosol DDT showed the following relative order of tolerance at the LD₅₀ level: Illinois 4.5, California 3.7, New Jersey 3.3, New Hampshire 3.0, and laboratory 1. After eight months of rearing as inbred cultures, devoid of exposure to any toxicants, all strains except the Illinois showed a marked decline in tolerance. Following an additional six months of similar inbred rearing, the Illinois strain had retained its original high level of tolerance. Prolonged maintenance of a static level of tolerance within an inbred tolerant strain,

devoid of exposure to the conditioning toxicant, does not seem probable.

Origin of tolerance, i.e., whether induced by field applications or by artificial laboratory selection, did not appear to influence the pattern of tolerance transmission. Apart from the actual level of filial tolerance expressed, there was no difference in the pattern of tolerance transmission, whether emanating from crosses between two tolerant strains or a tolerant and susceptible strain. Secondary tolerance to DDT expressed by the BHC-tolerant strain and primary tolerance to DDT expressed by the remaining strains appeared similarly transmitted.

When the progeny of crosses between tolerant and susceptible strains were backcrossed to the tolerant parent, the level of tolerance in the offspring was progressively increased with each successive backcross. A nearly symmetrically divergent pattern of tolerance evolved from equivalent backcrosses to the susceptible parent, the level of filial tolerance being progressively reduced with each such successive backcross.

Longer than normal life cycles in the subject DDT-tolerant strains were apparently derived from longer larval stages of development.

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BIOLOGICAL ACTIVATION OF SODIUM 2-(2,4-DICHLORO-PHENOXY)ETHYL SULFATE¹

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Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate² as a pre-emergence treatment for control of weeds (13) is a valuable addition to the plant growth substances which are structurally related to 2,4-dichlorophenoxyacetic acid (2,4-D). The material is inherently inert so that it does not injure foliage which is exposed to spray drift. Therefore, there is little opportunity for the devastating losses encountered from improper use near sensitive crops as in the case of 2,4-D. It does become active in destroying weed seedlings when incorporated into the soil, so it has been a useful herbicide in established crops such as asparagus or strawberries. The compound also has definite possibilities as a pre-emergence soil treatment in fields seeded to wheat, oats, rice, and similar crops which are resistant to 2,4-D.

The mechanisms by which an inactive material such as sodium 2-(2,4-dichlorophenoxy)ethyl sulfate becomes herbicidal is of considerable interest. Circumstantial evidence has been obtained that the material is acted upon by soil microorganisms since the compound is not effective in sterile soil (14). Carroll (7) has shown that the material may be readily hydrolyzed to 2-(2,4-dichlorophenoxy)ethanol and sodium acid sulfate in aqueous preparations at pH values of 3.0 to 4.0.

The present investigation was concerned with the rate of conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to active ingredients in soils at hydrogen-ion concentrations that would preclude acid hydrolysis. In addition, the plant growth regulating activity of 2-(2,4-dichlorophenoxy)ethanol and certain related compounds was compared with that of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and 2,4-D. These preliminary studies led to an investigation of the microorganisms responsible for the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to active ingredients, the metabolic processes of the microorganism responsible for the activation, and the end-products responsible for the destruction of weed seedlings. Data are presented on the particular role of *Bacillus cereus* var. *mycoides* (Flügge) Smith, Gordon, and Clark in converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to 2-(2,4-dichlorophenoxy)ethanol, an active plant growth regulator. The conversion of 2-(2,4-dichlorophenoxy)-ethanol to 2,4-D in soil was also demonstrated.

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² This chemical is known as CRAG Herbicide 1 of the Carbide and Carbon Chemicals Company.

REVIEW OF THE LITERATURE

SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was first described as a herbicide in 1949 (13). As a result of its unusual properties, appreciable interest in this compound has developed within a short period. Carroll (7) reported that acid hydrolysis of the compound in solutions of low pH results in the formation of 2-(2,4-dichlorophenoxy)ethanol and sodium acid sulfate. A short note was published by the author (23) indicating that the compound is also readily converted to an active form in soils of low pH values. Activation does not occur in sterile soil, however, if the hydrogen-ion concentration is above pH 5.5, whereas in nonsterile soil the conversion to an active compound occurs in a pH range of 4.0 to 7.0. At a soil pH of 8.0 the compound is inactive in suppressing cucumber primary root elongation. These results will be presented and discussed more fully in the present paper.

One of the hydrolysis products of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 2-(2,4-dichlorophenoxy)ethanol, has a high order of plant growth regulator activity if inhibition of cucumber root elongation is used as a criterion of activity. This chemical is active in producing epinastic responses in tomato plants when applied to foliage. However, if solutions of the compound are applied to soil of potted tomato plants or to excised tomato stems, the responses are considerably greater. It was suggested by Carroll (7) that 2-(2,4-dichlorophenoxy)ethanol does not travel rapidly downward in the phloem, whereas it travels upward in the xylem at a much greater rate.

MICROBIAL STUDIES WITH PLANT GROWTH REGULATORS

The hormone-types of herbicides such as 2,4-D come into contact with the soil when applied to foliage or when used as pre-emergence treatments for seeded fields. Their effects on the microorganisms in the soil and, conversely, the influence of the microorganisms on the stability of herbicides are vitally important considerations.

One of the earliest studies of the effect of 2,4-D on microorganisms (22) indicated that 0.02 per cent 2,4-D or its sodium salt with or without 0.5 per cent Carbowax in potato dextrose agar had a retarding effect on the growth of *Bacillus subtilis*, *Aerobacter cloacae*, *Staphylococcus aureus*, and *Phytomonas tumefaciens*. No detrimental effects were noted on the growth of *Fusarium* sp. or *Penicillium* sp. In general, under normal rates of 2,4-D application as a herbicide, the effect upon soil microorganisms is negligible (6, 8, 15, 21).

The rate of inactivation of 2,4-D in soil has been studied since this compound was first employed as a herbicide. Soil microbes were suggested as the agents responsible for decomposition of 2,4-D in soil (5, 9), and recent

work has substantiated the earlier theories (1, 2, 11, 19). Several workers have shown that 2,4-D will not decompose in autoclaved soil (5, 9, 11). Soil perfusion data (1, 2) have revealed that the kinetics of 2,4-D breakdown in soil follow a definite pattern divided into three phases: (a) initial adsorption to soil colloids, (b) a lag phase with little or no decomposition, and (c) a final stage of rapid, complete detoxication. Bacteria capable of decomposing 2,4-D have been isolated in pure culture and have been shown to be related to the *Bacterium globiforme* group (1). The chemical nature of the decomposition products of 2,4-D in soil has not been described. Audus (2) has suggested 2,4-dichlorophenol as a possible product of microbial activity.

The action of soil microbes on sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was suggested in an earlier description of the compound (14). *Bacillus cereus* var. *mycoides* was recently reported capable of converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator (24). The details of this bacterial action were not presented in the preliminary paper, but will be discussed more fully herein.

While the present paper was in preparation Audus (3) published a short note indicating that sodium 2-(2,4-dichlorophenoxy)ethyl sulfate is converted to 2,4-dichlorophenoxyacetic acid by unidentified soil microorganisms. Pathways of the conversion process were not discussed.

MATERIALS AND METHODS

CUCUMBER ROOT SUPPRESSION TEST

Root suppression of cucumber seedlings (20) was employed throughout the investigation as a principal criterion for plant growth regulating activity. With certain modifications which are discussed under a separate heading, this test involved the following procedures. Twenty-five cucumber (*Cucumis sativus* L. var. Davis' Perfect) seed were placed in contact with 5 ml. of aqueous or emulsified preparations of the test chemical on filter paper in nine-inch Petri dishes. The cultures were maintained at 25° C. for five days after which root lengths were measured. Duplicate or triplicate series were included for each treatment.

TOMATO PLANT RESPONSES

Several responses on tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) were used to compare the activity of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 2-(2,4-dichlorophenoxy)ethanol, and 2,4-D following the recent work of Hitchcock and Zimmerman (12). This method consisted of applying emulsions or aqueous solutions of the test chemicals at concentrations of 1.0, 0.1, 0.01, 0.001, and 0.0001 per cent to soil in which young tomato plants 7 to 9 cm. in height were growing. Readings were made for leaf or stem curvatures after 2, 5, and 24 hours. Relative degree

of leaf modification was recorded after three days. Increases in height of test plants were taken seven days following treatment. Maximum curvature, modification, or proliferation responses were rated + + +, and minimum responses as +. Magnitude of stem curvature was obtained by summing the responses given by each replicate (i.e. + +, + +, + + = 6) and multiplying by a factor (5 or 6).

QUANTITATIVE AND QUALITATIVE DETERMINATIONS OF 2-(2,4-DICHLOROPHENOXY)ETHANOL AND 2,4-D IN SOILS TREATED WITH SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

The production of 2-(2,4-dichlorophenoxy)ethanol in soils treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was measured by modifying a quantitative method recently developed for analyzing plant tissues by chemists of the Carbide and Carbon Chemicals Company.³ Typical experiments involved adding 0.02 to 0.80 mg. of sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate to sandy loam soils of pH 5.7 to 5.9, incubating them for intervals of 15 minutes to two hours and destroying the microorganisms and their enzymes by autoclaving at 15 pounds steam pressure for 30 minutes. The soil was extracted by three successive washings with 100 ml. of chloroform per 20 g. of soil. Extracts were concentrated to 10 ml. on a steam bath, and were then cooled and treated with 10 ml. of a 2 per cent solution of chlorosulfonic acid in chloroform. After swirling vigorously, the solution was allowed to stand for two minutes and then was washed with 25 ml. of water in a 250-ml. separatory funnel. After ten minutes the chloroform fraction was removed and discarded, while the aqueous fraction was filtered through paper (Whatman No. 1) into a clean separatory funnel containing 50 ml. of chloroform and 25 ml. of methylene blue chloride solution (0.050 g. of Eastman Kodak reagent grade methylene blue chloride indicator in one liter of distilled water to which was added 10 ml. of concentrated sulfuric acid and 50 g. of anhydrous sodium sulfate). The contents of each funnel were shaken and the layers allowed to separate and stand at room temperature for 15 minutes. The lower layers were drawn off into Klett colorimetric test tubes and placed in a Klett-Summerson colorimeter after the instrument was first adjusted with an appropriate blank sample. A typical calibration curve is shown in Figure 1.

The principles involved in the above procedure are as follows: reaction of the chloroform extract with dilute chlorosulfonic acid solution gives the 2-(2,4-dichlorophenoxy)ethyl hydrogen sulfate, which can be re-extracted with water and added to a two-phase system of chloroform and water containing methylene blue chloride indicator in the aqueous phase. The

³ Unpublished data. The author is indebted to the Carbide and Carbon Chemicals Company for making this method available.

colored complex which is formed from the 2-(2,4-dichlorophenoxy)ethyl hydrogen sulfate and the indicator is extracted quantitatively into the chloroform layer, and the intensity of color is measured with the colorimeter.

A method of analyzing for 2,4-D has been reported previously by Freed (10). This method, and that of Marquardt and Luce (16), have been modified for the present study. Twenty-gram samples of soil were treated with 0.02 mg. to 0.80 mg. of either sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate or 2-(2,4-dichlorophenoxy)ethanol. Each soil-chemical mixture was incubated at 25° C. for 30, 60, 120, and 180 minutes. After each

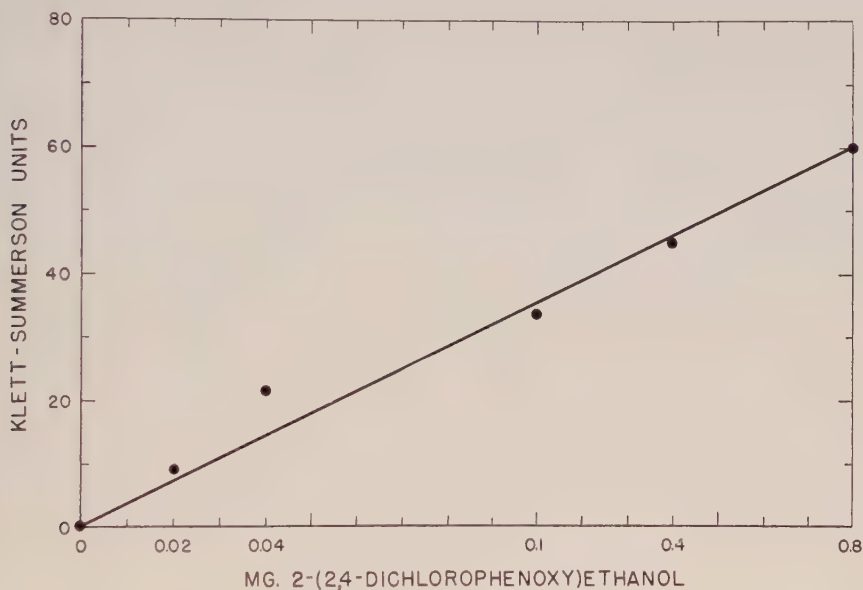


FIGURE 1. Calibration curve for 2-(2,4-dichlorophenoxy)ethanol extracted from soil.

incubation period had expired, the soil-chemical mixtures were autoclaved for 30 minutes at 15 pounds steam pressure. Each soil sample was acidified with HCl to pH 3.0. Chloroform was added in 50-ml. portions to each acidified soil, and the samples agitated thoroughly with a mechanical glass stirrer. The soil extract was passed through Whatman No. 1 filter paper, and the resulting filtrate concentrated to 0.1 ml. on a steam bath. Five ml. of a chromotropic acid solution (0.25 g. of 1,8-dihydroxynaphthalene-3,6-disulfonic acid in 75 ml. methanol) was added to the soil filtrate. To this was added 5 ml. of concentrated sulfuric acid. The sample was stirred thoroughly and placed in an oven at 130° to 135° C. for 20 minutes. Duplicate samples were included for all treatments. The presence of 2,4-D was indicated by the development of a deep wine-purple color. Negative tests

were obtained when 2-(2,4-dichlorophenoxy)ethanol or sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was subjected to this colorimetric analysis. The mechanism of this color reaction is not known, but investigations are under way to determine its nature (10).

SOIL DILUTION—ISOLATION TECHNIQUES

Isolation of soil organisms capable of converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active form was accomplished by means of soil dilution plates. These were prepared with 10-gram samples of sandy loam soil (pH 5.5 to 7.0) which were previously treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (100 p.p.m.) and incubated for seven days at 21° C. Each soil sample was added to 200 ml. of sterile water in 500-ml. Erlenmeyer flasks, which were then shaken for 30 minutes on an automatic "wrist-action" shaker. The soil-water mixture in each flask was allowed to stand for ten minutes after removal from the shaker.

Serial dilutions were prepared from the original 1:20 dilutions. Differential media were employed consisting of soil agar (23) for actinomycetes, nutrient agar for bacteria, and potato dextrose agar for fungi. One ml. of a 1:100,000 dilution was added to each of three sterile Petri dishes and cool agar poured over the dilution. All cultures were incubated at room temperature (20° to 25° C.). After 24 hours, transfers of bacterial colonies appearing on the Petri dish cultures were made to test tube slants containing nutrient agar. After four or five days, fungus and actinomycete colonies were transferred to test tube slants of potato dextrose and soil agar respectively. When the soil organisms were well established in test tube slants, each of the isolates was tested for its ability to convert sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to its active form in sterilized soil by means of the cucumber root suppression test.

EXPERIMENTAL RESULTS

NATURE OF ACTIVATION OF SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE IN SOILS

Effect of hydrogen-ion concentration on the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator in soil. The conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active composition in sterile and nonsterile sandy loam soils adjusted to pH 3.0, 4.0, 5.5, 6.0, 7.0, and 8.0 by adding H_3PO_4 or NaOH was investigated. Soil samples were distributed in ten Petri dishes, five of which were autoclaved at 15 pounds steam pressure for one hour. A comparable number were retained as nonsterile controls. Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was added to each dish at the rate of 5 p.p.m. of soil and 25 cucumber seeds were placed immediately on the soil in each dish. Cultures were in-

cubated at 25° C. for five days. At the end of this time lengths of primary roots of germinated seeds were measured.

Conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active form occurred in sterile soil only at pH values 3.0 and 4.0 (Fig. 2 A). In nonsterile soil the conversion took place in the pH range of 3.0 and 7.0, but not at pH 8.0 (Fig. 2 B). Previous reports had indicated that the compound was inactive in sterile soil (13, 14). However, the sterile soils used were not acid in reaction. Cucumber root suppression obtained in nonsterile soils of neutral pH (Fig. 2 B) eliminated the possibility for acid hydrolysis in these soils.

Effect of temperature on the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator in soil. The effect of temperature upon the soil activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was included for study in preliminary tests. Fifty grams of sandy loam soil (pH 6.0) were treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate at 5 p.p.m. Duplicate samples were stored in Petri dishes at constant temperatures of 1°, 5°, 10°, 15°, 20°, and 25° C. for a period of 48 hours. After the incubation period, each sample was autoclaved for one hour at 15 pounds pressure. Cucumber seed were then placed in contact with the soil-chemical mixture for five days at 25° C., and primary root elongation measured at the end of this period.

The conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active form was most pronounced at 25° C. (Fig. 3). There was a progressive decrease in soil activation as the temperature of incubation was decreased. However, there was considerable swelling and inhibition of primary root elongation even in the soil-chemical samples stored at 1° C. There was no root suppression in control soil or in solutions of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate stored at any of the incubation temperatures.

Time required for conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active form in soil. The length of time required for the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active herbicide in soil was investigated by incubating the chemical for given lengths of time in soil, subjecting the soil to autoclaving, and then using the cucumber primary root suppression test as a criterion of activation. Twenty grams of sandy loam soil (pH 6.0) in Petri dishes were treated with 5 p.p.m. of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and incubated at 25° C. for 15-minute intervals up to three hours. At the end of each incubation period, the soil samples were autoclaved. Any breakdown of the chemical to its active form should have occurred before autoclaving. When each sample had cooled sufficiently following autoclaving, it was planted in duplicate with 25 cucumber seed. Primary root lengths were measured after five days.

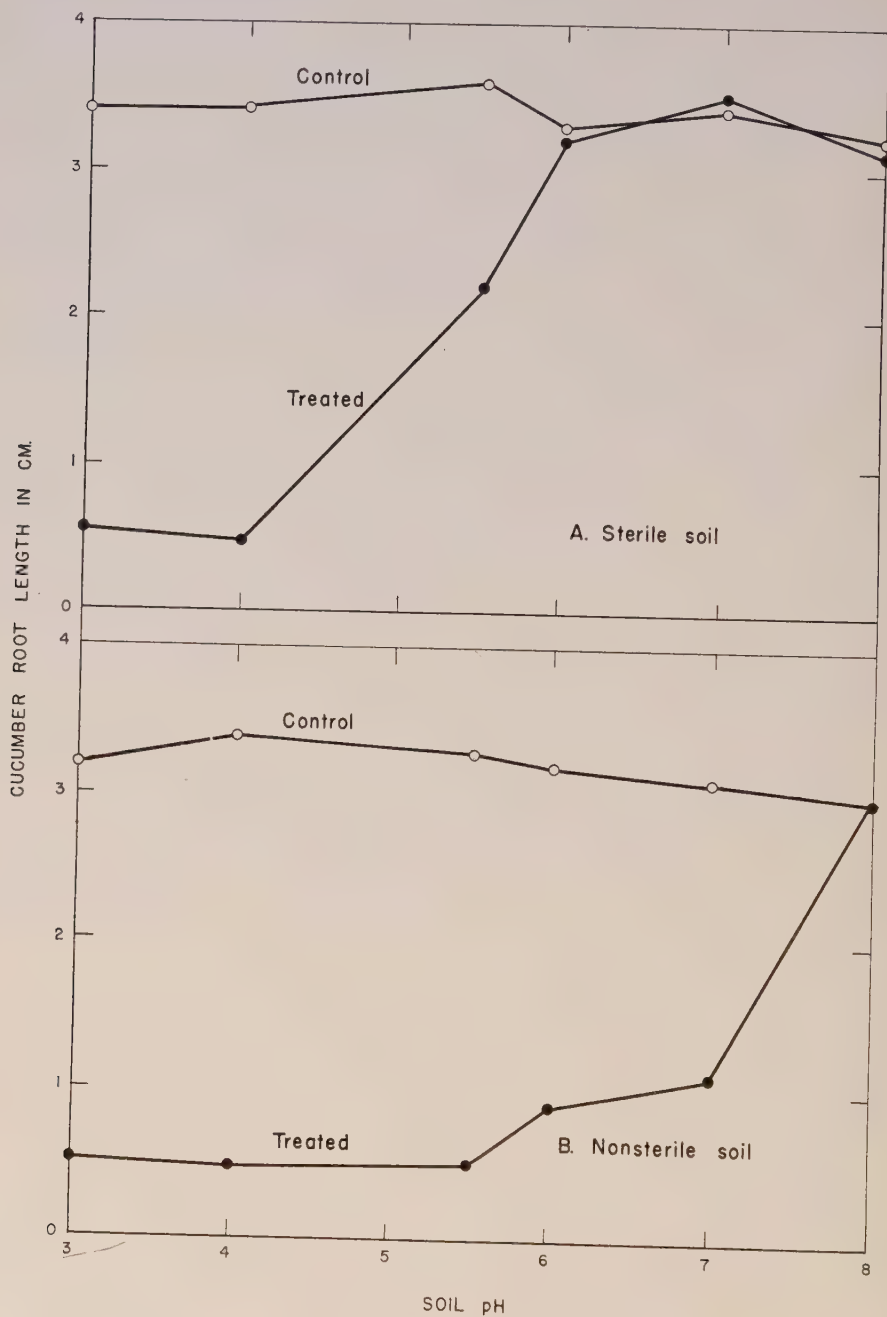


FIGURE 2. Effect of hydrogen-ion concentration on cucumber root suppression by sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (5 p.p.m.) in (A) sterile soil; (B) nonsterile soil.

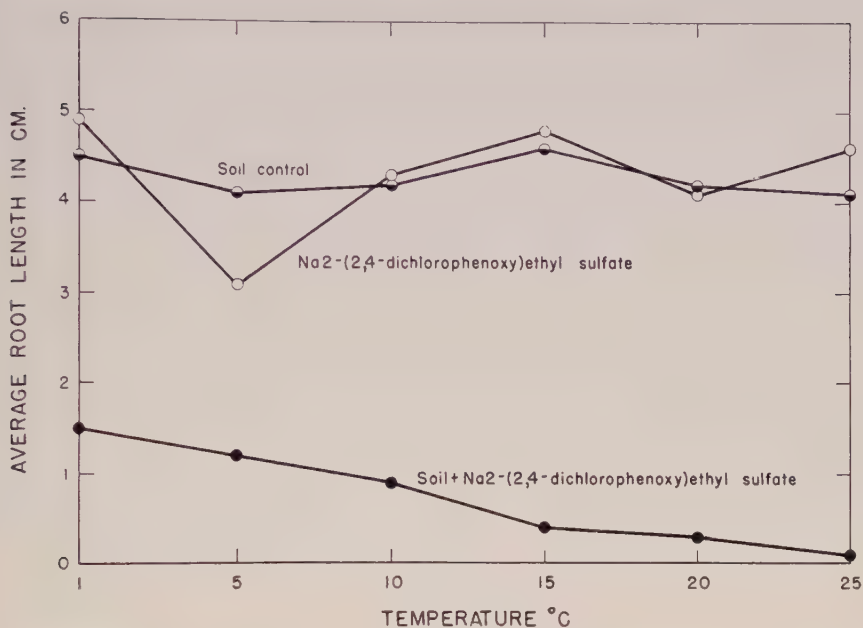


FIGURE 3. Effect of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate at 5 p.p.m. upon cucumber root suppression in soils stored at 1° to 25° C.

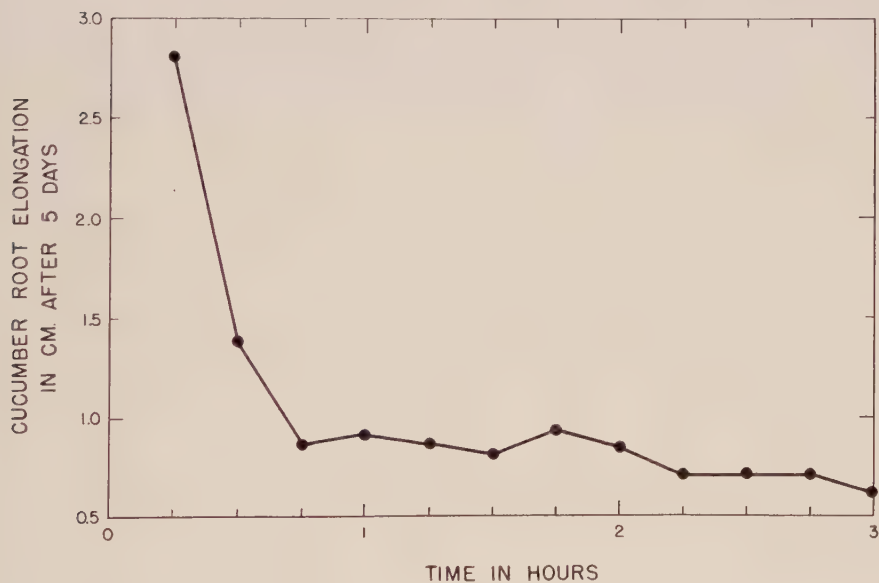


FIGURE 4. Length of time required for conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active form in soil.

Conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in soil to an active compound capable of suppressing the growth of primary roots took place within 45 minutes (Fig. 4). Slight swelling and root inhibition were noted in samples which had been incubated for 30 minutes before autoclaving, but none in 15-minute incubation samples. Presumably, microbial action accounted for activation in all the soils used in the time experiments, since previous tests had shown that in sterile soil at pH 6.0 the conversion to an active form does not occur. In nonsterile soils of the same pH, sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was activated.

COMPARISON OF SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE
WITH CERTAIN STRUCTURALLY RELATED COMPOUNDS

Cucumber root suppression. One phase of the investigation consisted in comparing the relative activity of (a) 2-(2,4-dichlorophenoxy)ethanol, (b) 2,4-D, (c) 2-(2,4-dichlorophenoxy)ethyl benzoate, (d) bis[2-(2,4-dichlorophenoxy)ethyl] oxalate, and (e) sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. Structural formulae for these five chemicals appear below. The 2-(2,4-dichlorophenoxy)ethanol equivalent for each of the last three of these compounds appears to the right of its structural formula, where R stands for 2,4-dichlorophenyl.

(a) $R-O-CH_2CH_2-OH$	
(b) $R-O-CH_2-COOH$	
	2-(2,4-Dichlorophenoxy)ethanol equivalents in per cent
(c) $R-O-CH_2CH_2-O-CO-C_6H_5$	66.2
(d) $R-O-CH_2CH_2-O-C=O$ $R-O-CH_2CH_2-O-C=O$	89.0
(e) $R-O-CH_2CH_2-O-SO_3Na$	67.2

Twenty-gram samples of sandy loam soil (pH 6.0) in Petri dishes were treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 2-(2,4-dichlorophenoxy)ethanol, 2-(2,4-dichlorophenoxy)ethyl benzoate, and bis[2-(2,4-dichlorophenoxy)ethyl] oxalate at rates equivalent to 5.0, 0.5, and 0.05 p.p.m. of 2-(2,4-dichlorophenoxy)ethanol. Five samples were autoclaved for 30 minutes at 15 pounds pressure, while a comparable number were retained as nonsterile controls. Each chemical in aqueous solution was also autoclaved in Petri dishes containing filter paper and similar preparations were not autoclaved. Cucumber seed were placed in contact with the soil or filter paper in each dish.

Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate suppressed cucumber root lengths only in nonsterile soils (Table I). 2-(2,4-Dichlorophenoxy)ethyl benzoate behaved similarly to sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in that it gave greater root suppression in nonsterile soils. However, root suppression was evident in sterile soils as well, indicating that the chemical possesses innate plant growth regulating activity. When com-

TABLE I

ACTIVITY OF 2-(2,4-DICHLOROPHENOXY)ETHANOL AND THREE RELATED COMPOUNDS IN SUPPRESSING ROOT GROWTH OF CUCUMBER SEEDLINGS IN STERILE AND NON-STERILE SOIL AND IN AQUEOUS PREPARATIONS ON FILTER PAPER

Chemical	Concn., p.p.m.	Root length of seedlings in cm. on			
		Moist paper		Moist soil	
		Sterile	Nonsterile	Sterile	Nonsterile
2-(2,4-Dichlorophenoxy)- ethanol	5	0.7	0.6	0.6	0.4
	0.5	0.8	1.0	0.9	0.6
	0.05	1.5	1.5	1.4	0.6
Sodium 2-(2,4-dichloro- phenoxy)ethyl sulfate	5	4.7	3.1	2.6	0.5
	0.5	5.5	2.7	3.0	0.8
	0.05	4.8	3.4	3.3	1.6
2-(2,4-Dichlorophenoxy)- ethyl benzoate	5	0.8	1.0	1.2	0.5
	0.5	2.4	2.2	2.0	1.1
	0.05	3.2	2.7	2.3	2.1
Bis[2-(2,4-dichlorophenoxy)- ethyl] oxalate	5	0.4	0.8	0.7	0.4
	0.5	1.1	0.9	1.1	0.9
	0.05	2.5	2.4	1.9	2.1
None		3.6	3.4	3.4	3.4

pared with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in sterile soils, 2-(2,4-dichlorophenoxy)ethyl benzoate possesses greater root inhibitive properties.

Bis[2-(2,4-dichlorophenoxy)ethyl] oxalate was more effective than either sodium 2-(2,4-dichlorophenoxy)ethyl sulfate or 2-(2,4-dichlorophenoxy)ethyl benzoate in sterile soil. The sterilized chemical in absence of soil was as active as the nonsterile soil treatment indicating that micro-organisms are not a factor in the action of bis[2-(2,4-dichlorophenoxy)-ethyl] oxalate.

Cucumber root suppression was evident in 2-(2,4-dichlorophenoxy)-ethanol treatments at the three concentrations employed. The results presented in Table I indicate that this chemical is more active in suppressing cucumber root elongation than any of the compounds used in these tests.

The qualitative responses of cucumber primary roots to 2-(2,4-dichlorophenoxy)ethanol are similar to those obtained with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, bis[2-(2,4-dichlorophenoxy)ethyl] oxalate, 2-(2,4-dichlorophenoxy)ethyl benzoate, or 2,4-D. These responses take the form of extreme swelling and marked stunting of the primary root tip.

Tomato plant responses. Responses on tomato were also used to assay the activity of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 2-(2,4-dichlorophenoxy)ethanol, and 2,4-D. The triethanolamine salt of 2,4-D was prepared in aqueous solution at concentrations of 1.0, 0.1, 0.01, 0.001, and 0.0001 per cent. Two formulations of 2-(2,4-dichlorophenoxy)ethanol were employed. The first (Formulation 1), prepared at Mellon Institute, consisted of 50 parts 2-(2,4-dichlorophenoxy)ethanol, 22.5 parts xylene, 22.5 parts isophorone (3,5,5-trimethylcyclohexene-2-one-1) and 5 parts emulsifier 75H-14-S (polyglycol monostearate). The second formulation (Formulation 2) consisted of 1.0 per cent 2-(2,4-dichlorophenoxy)ethanol in 10 per cent Emulfor in water (polyglycol ester of long chain fatty acids). Concentrations of 1.0, 0.1, 0.01, 0.001, and 0.0001 per cent were prepared of each formulation. Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was prepared in aqueous solution at similar concentrations. Ten ml. of chemical at the above concentrations were added to soil in which the tomato plants were growing, so that 100.0, 10.0, 1.0, 0.1, or 0.01 mg. of chemical were added to each four-inch pot.

Similar quantities of the above formulated chemicals were added to 100 ml. H₂O in 125-ml. Erlenmeyer flasks. Young tomato plants were removed from soil, the roots washed thoroughly in tap water, and transferred to the flasks containing the chemical in emulsion.

Comparisons of the three compounds in soil in which tomato plants were growing with the aqueous preparations of the chemicals to which rooted plants were transferred are given in Table II. Growth increments of treated tomato plants are listed in Table III.

Initial stem curvatures on tomato plants treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate at the rate of 1.0 mg. per four-inch pot appeared within two hours. Below this concentration no curvatures were obtained even after 24 hours. Leaf modification was present after six days on plants which had not given visible curvatures (that is, at concentrations of 0.1 mg. per pot). After six days, plants receiving 100 mg. were dead and those receiving 1.0 to 10.0 mg. of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate exhibited proliferation. Plants transferred from soil to aqueous preparations of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate showed no hormone responses.

There was a decided difference in the responses induced by 2-(2,4-dichlorophenoxy)ethanol dependent upon the formulation employed. Formulation 2 gave initial stem curvatures at concentrations of 100, 10,

TABLE II

COMPARATIVE ACTIVITY OF SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE,
2-(2,4-DICHLOROPHENOXY)ETHANOL, AND 2,4-D FOR INDUCING
RESPONSES ON TOMATO PLANTS

Treatments	Mg. per pot	Response of plants in soil					Response of plants in water after 2 hours, stem curvature
		Stem curvature after			After 6 days		
		2 hrs.	5 hrs.	24 hrs.	Leaf modifica- tion	Prolifera- tion	
Sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate	100.0	+	+++	+++	k	k	o
	10.0	+	+++	+++	o	+	o
	1.0	+	+++	+++	o	+	o
	0.1	o	o	o	+	o	o
	0.01	o	o	o	o	o	o
	0.0	o	o	o	o	o	o
2-(2,4-Dichlorophenoxy)ethanol (Formulation 1)	100.0	k	k	k	k	k	++
	10.0	k	k	k	k	k	+
	1.0	+	+++	+++	o	+	+
	0.1	+	+++	+++	++	+++	+
	0.01	o	o	+	+	o	o
	0.0	o	o	o	o	o	o
2-(2,4-Dichlorophenoxy)ethanol (Formulation 2)	100.0	+	+++	+++	k	k	++
	10.0	+	+++	+++	o	+	+
	1.0	+	+++	+++	o	++	+
	0.1	o	+	++	+++	o	o
	0.01	o	o	o	o	o	o
	0.0	o	o	o	o	o	o
2,4-D	100.0	++	+++	+++	k	k	++
	10.0	++	+++	+++	o	+	+
	1.0	+	+++	+++	o	+	+
	0.1	+	+	+	+++	+	+
	0.01	o	o	o	o	o	+
	0.0	o	o	o	o	o	o

o = No response; + = minimal response; +++ = maximal response, k = plant killed.

and 1 mg. per pot after two hours, and at 0.1 mg. after four hours. Systemic stem burn was caused by Formulation 1 at concentrations of 100 and 10 mg. per pot after two hours. At 1.0 and 0.1 mg. there were stem curvatures after two hours, while at 0.01 mg. slight curvatures were present after 24 hours.

The triethanolamine salt of 2,4-D caused initial stem curvatures after two hours at concentrations of 100, 10.0, and 1.0 mg. After six days, leaf modification was present in plants treated with 0.1 mg. 2,4-D. Proliferation was severe at 1.0 mg. and slight at 10.0 mg.

On the basis of leaf modification on tomato plants in soil treated with Formulation 2 of 2-(2,4-dichlorophenoxy)ethanol and 2,4-D, both compounds were of equal activity, whereas Formulation 1 of 2-(2,4-dichlorophenoxy)ethanol was more active than 2,4-D. In contrast, 2,4-D was more

TABLE III
EFFECT OF SOIL TREATMENTS WITH SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE, 2-(2,4-DICHLOROPHENOXY)ETHANOL, AND 2,4-DICHLOROPHENOXYACETIC ACID ON GROWTH OF TOMATO PLANTS

Treatments	Increase in height (cm.) according to quantity of chemical in mg.				
	100.0	10.0	1.0	0.1	0.01
Sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate	k	1.0	4.5	5.9	6.6
2-(2,4-Dichlorophenoxy)ethanol (Formulation 1)	k	k	0.5	2.4	5.3
2-(2,4-Dichlorophenoxy)ethanol (Formulation 2)	k	0.7	3.4	6.6	6.5
2,4-D (Triethanolamine salt)	k	0.9	2.7	5.6	7.0
Untreated			6.5		

k = Plants killed.

active than Formulation 2 of 2-(2,4-dichlorophenoxy)ethanol, and of equal activity to Formulation 1 of 2-(2,4-dichlorophenoxy)ethanol for inducing stem curvatures on tomato plants.

Aqueous preparations of 2-(2,4-dichlorophenoxy)ethanol (Formulation 2) caused stem curvature only at concentrations of 1 mg. or more, while in soil pronounced curvatures were obtained at 0.1 mg. (Fig. 5).

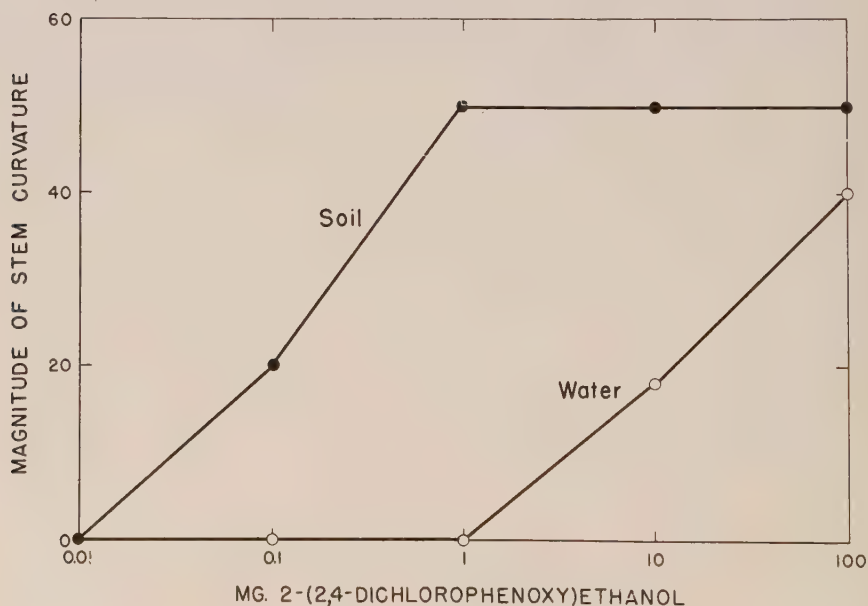


FIGURE 5. Effect of 2-(2,4-dichlorophenoxy)ethanol at five concentrations in inducing stem curvature of tomato plants in soil and water solutions.

Stem curvatures were induced on tomato plants in the absence of soil by 2,4-D at concentrations of 0.01 mg., while in soil 0.1 mg. was necessary to produce this response. Adsorption of the chemical to soil colloids or microbial decomposition of 2,4-D may have accounted for this difference. Curvature responses of test plants were masked at higher concentrations of 2,4-D because of severe wilting.

QUANTITATIVE AND QUALITATIVE DETERMINATIONS OF 2-(2,4-DICHLOROPHENOXY)ETHANOL AND 2,4-D IN SOILS TREATED WITH SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

The results obtained from quantitative analyses of soils treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (Table IV) reveal a rapid conversion of the inactive compound to the active 2-(2,4-dichlorophenoxy)-ethanol. After two hours at an initial concentration of 0.02 mg. sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 0.02 mg. of 2-(2,4-dichlorophenoxy)-ethanol was found. As the concentration of sodium 2-(2,4-dichloro-

TABLE IV
RATE AT WHICH 2-(2,4-DICHLOROPHENOXY)ETHANOL WAS PRODUCED
IN MG. IN SANDY LOAM SOILS TREATED WITH SODIUM
2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

Concn. of sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate, mg.	Incubation period before autoclaving, in minutes							
	15	30	45	60	75	90	105	120
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.02	0.0	0.017	0.018	0.020	0.020	0.020	0.020	0.020
0.04	0.0	0.030	0.070	0.040	0.049	0.040	—	0.050
0.10	0.0	0.023	0.040	0.039	0.049	0.065	0.065	0.065
0.20	0.010	0.040	0.068	0.075	0.084	0.079	0.084	0.092
0.40	0.0	0.066	0.103	0.240	0.125	0.340	—	0.250
0.80	0.0	0.047	0.075	0.075	0.400	0.400	0.380	0.450

phenoxy)ethyl sulfate was increased in soil, the percentage conversion to 2-(2,4-dichlorophenoxy)ethanol decreased so that at the 0.80 mg. concentration, only 0.45 mg. of 2-(2,4-dichlorophenoxy)ethanol was found after 120 minutes.

Qualitative analyses for 2,4-D in soils treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and 2-(2,4-dichlorophenoxy)ethanol are presented in Table V. The presence of 2,4-D in soils treated with 2-(2,4-dichlorophenoxy)ethyl sulfate was detected after 120 and 180 minutes, but negative tests were obtained in previously sterilized soils or if the incubation period lasted 30 to 60 minutes. The intensity of color was greater in samples incubated for longer time periods, but it was not possible to translate this color difference to quantitative terms.

In soils treated with 2-(2,4-dichlorophenoxy)ethanol positive color reactions for 2,4-dichlorophenoxyacetic acid were obtained in soil samples containing 0.20 mg., 0.40 mg., and 0.80 mg. 2-(2,4-dichlorophenoxy)-

TABLE V

DETECTION OF 2,4-D IN SOILS TREATED WITH SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE AND 2-(2,4-DICHLOROPHENOXY)ETHANOL

Chemical added to soil, mg.	Presence of 2,4-D after incubation in soil, in minutes		
	60	120	180
Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate			
0.02	o	+	+
0.04	o	+	+
0.10	o	+	+
0.20	o	+	+
0.40	o	+	+
0.80	o	+	+
2-(2,4-Dichlorophenoxy)ethanol			
0.02	o	+	+
0.04	o	+	+
0.10	o	+	+
0.20	+	+	+
0.40	+	+	+
0.80	+	+	+

2,4-D present = +, absent = o.

ethanol after 60 minutes. These results indicate that when soil is treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate two highly active plant growth regulators are formed.

ISOLATION AND IDENTIFICATION OF SOIL MICROORGANISMS ACTIVATING SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

Conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator by soil isolates. Bacteria were found to be most active in converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to its growth-regulant form (24). In the initial experiments no effort was made to identify each isolate. However, if the isolate exhibited activity it was then taxonomically determined. Representative data from a portion of the soil isolate-herbicide activation tests appear in Table VI.

The responses of cucumber primary roots to the activated form of the herbicide induced by pure cultures of B-1 and B-11 were the typical swelling and marked stunting of growth. These qualitative manifestations confirmed the quantitative root length measurements. Cultures B-1 and B-11 were identified as *Bacillus cereus* var. *mycoides*. Standard bacteriological procedures were followed for identification of the organism (4). Results of these may be summarized as follows: gram-positive rods 1.0 to 1.2 μ by 3.0 to 5.0 μ ; rhizoidal growth on nutrient agar slants; loss of rhizoidal characteristic in nutrient broth; gelatin liquefaction rapid; negative fermentation tests on xylose and arabinose; positive on sucrose and glucose;

positive Voges-Proskauer reaction; positive starch hydrolysis; nitrites produced from nitrates.

Although other soil microorganisms were isolated (24) which also activated sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, *B. cereus* var. *mycoides* was selected as the organism with which to conduct more detailed experiments concerning the activation mechanism.

TABLE VI
EFFECT OF SOME BACTERIAL ISOLATES IN ACTIVATING SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE IN STERILE SOIL

Sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate, 5 p.p.m.	Bacterial isolate	Cucumber root length,* cm.
Present	None	2.1
Absent	None	2.2
Present	B-1	0.5
Absent	B-1	2.1
Present	B-11	0.7
Absent	B-11	2.5
Present	B-10	2.1
Absent	B-10	2.5

* Average primary cucumber root length for 50 seeds.

The action of *Bacillus cereus* var. *mycoides* on sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. Cell-free filtrates of nutrient broth in which *Bacillus cereus* var. *mycoides* had been grown for 24 hours were obtained by passing the cultures through a Morton bacteriological filter (17, 18). The resulting filtrate was tested for sterility by transfer of a drop to a nutrient agar slant. These cell-free preparations were then tested for their action on sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. Results obtained in such an experiment are listed in Table VII.

Since the pH of the filtrates used was above 7.0, acid hydrolysis obviously was not involved in the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to its active form. The chemical was converted to an active form when it came into contact with filtrates of nutrient broth cultures of *B. cereus* var. *mycoides*, but conversion did not occur if these filtrates were previously autoclaved for 15 minutes at 15 lb. pressure.

More detailed experiments were performed to determine whether *B. cereus* var. *mycoides* could convert sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to the active herbicide 2-(2,4-dichlorophenoxy)ethanol. The organism was grown on the following synthetic medium.⁴

NH ₄ H ₂ PO ₄	1.0 g.
KCl	0.2 g.
MgSO ₄ · 7H ₂ O	0.2 g.
Glucose	1.0%
H ₂ O	1.0 l.
pH	7.0

⁴ The author is indebted to Dr. Francis E. Clark of Iowa State College for recommending this medium.

Glucose was sterilized by filtration before being added to the medium, since it was found to be bacteriostatic when autoclaved in the complete medium. *B. cereus* var. *mycoides* was grown in 100 ml. of the medium in 250-ml. Erlenmeyer flasks for 24 hours at 25° C. At the end of the incubation period the contents of each flask were passed through a Morton bacteriological filter, and the resulting filtrate tested for sterility. The following amounts of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in sterile

TABLE VII
EFFECT OF CELL-FREE FILTRATES OF *BACILLUS CEREUS* VAR. *MYCOIDES* IN
ACTIVATING SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

Treatment	Concn. of chemical, p.p.m.	Cucumber root length,* cm.
Filtrate+chemical	5	0.6
	0.5	1.0
	0.05	1.2
Chemical alone	5	2.3
	0.5	2.2
	0.05	2.3
Autoclaved filtrate+chemical	5	2.5
	0.5	2.3
	0.05	2.5
Filtrate alone	—	2.0
Water control	—	2.2

* Average primary cucumber root length for 50 seed.

aqueous solution were then added to each filtrate: 0.20, 0.40, and 0.80 mg. Filtrates containing sodium 2-(2,4-dichlorophenoxy)ethyl sulfate were autoclaved after 60, 90, and 120 minutes. (Inactivation of filtrates was also obtained by heating to 72° C.) Quantitative determinations for 2-(2,4-dichlorophenoxy)ethanol and qualitative tests for 2,4-D were made after autoclaving.

2-(2,4-Dichlorophenoxy)ethanol was detected after 60, 90, and 120 minutes in all filtrates of *B. cereus* var. *mycoides* to which sodium 2-(2,4-dichlorophenoxy)ethyl sulfate had been added (Table VIII).

In no case, however, was the rate of conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to 2-(2,4-dichlorophenoxy)ethanol so rapid as in nonsterile soil (Table IV). Qualitative tests for 2,4-D were negative in filtrates of *B. cereus* var. *mycoides* containing sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and incubated for periods up to one week.

The action of *Bacillus cereus* var. *mycoides* on 2-(2,4-dichlorophenoxy)-ethyl benzoate. The cucumber root suppression test was employed to measure the activation of 2-(2,4-dichlorophenoxy)ethyl benzoate by *B. cereus*

TABLE VIII

AMOUNT IN MG. OF 2-(2,4-DICHLOROPHENOXY)ETHANOL FORMED FROM SODIUM
2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE IN CELL-FREE
FILTRATES OF *B. CEREUS* VAR. *MYCOIDES*

Amount of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in filtrate, mg.	Time of incubation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in filtrate before autoclaving, minutes		
	60	90	120
0.20	0.04	0.07	0.08
0.40	0.06	0.20	0.20
0.80	0.06	0.38	0.42
0.0	0.0	0.0	0.0

var. *mycoides*. Five-gram samples of sandy loam soil were treated with 5.0, 0.5, and 0.05 p.p.m. of the chemical in Petri dishes and autoclaved for 30 minutes at 15 lb. pressure. When the soil samples had cooled they were inoculated with a 1.0-ml. suspension of *B. cereus* var. *mycoides* grown on nutrient agar slants. Twenty-five cucumber seed were placed in contact with the soil-herbicide-microbial mixture and incubated for five days at 21° C. Controls consisting of sterile soil, herbicide, and *B. cereus* var. *mycoides* were included. Primary root length measurements were taken after five days. Results appear in Table IX.

The presence or absence of *B. cereus* var. *mycoides* in soil containing 2-(2,4-dichlorophenoxy)ethyl benzoate had no effect on the suppression of cucumber root lengths by the chemical.

Arylsulfatase activity in Bacillus cereus var. *mycoides*. Arylsulfatase activity in soil-herbicide isolates of *B. cereus* var. *mycoides* was investigated by the following procedure: The organism was grown in nutrient broth medium containing 0.001 M potassium phenolphthalein disulfate. After one, three, and seven days incubation at 30° C., 1 N sodium hydroxide was added dropwise to the cultures. Phenolphthalein liberated by enzymic hydrolysis can thus be detected by red coloration produced by the addition of sodium hydroxide, if an organism produces arylsulfatase.

Results of repeated tests for arylsulfatase activity in *B. cereus* var. *mycoides* were negative.

TABLE IX

RELATIVE ACTIVITY OF 2-(2,4-DICHLOROPHENOXY)ETHYL BENZOATE IN THE
PRESENCE AND ABSENCE OF *B. CEREUS* VAR. *MYCOIDES* IN AUTOCLAVED SOIL

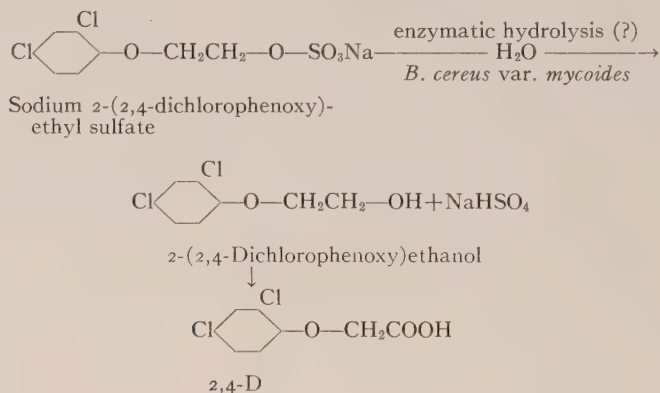
Concn. p.p.m. of 2-(2,4-dichlorophenoxy)-ethyl benzoate	Length of cucumber roots (cm.) when bacteria were	
	Present	Absent
5	1.5	1.3
0.5	2.3	2.4
0.05	2.6	2.6
0.0	3.6	3.4

Microbial action on 2-(2,4-dichlorophenoxy)ethanol. The detection of 2,4-D in soils treated with 2-(2,4-dichlorophenoxy)ethanol (Table V) suggested the possibility of microbial oxidation of the ethanol side chain to acetic acid. *Acetobacter aceti* (Kützing) Beijerinck was grown in 100 ml. nutrient broth containing 0.05, 0.5, or 5.0 p.p.m. of 2-(2,4-dichlorophenoxy)ethanol. Cultures were incubated at 30° C. Qualitative determinations for 2,4-D were made on samples withdrawn from each culture after one-, two-, three-, and five-day intervals.

Bacterial growth was heavy in nutrient broth cultures containing 0.05 p.p.m. 2-(2,4-dichlorophenoxy)ethanol, but sparse in cultures containing 0.5 p.p.m. of the chemical. There was no growth in broth cultures containing 5.0 p.p.m. of the compound. Tests for 2,4-D were negative in all samples.

DISCUSSION

The addition of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to soils results in the conversion of the compound to two highly active plant growth regulators. What previously has been described as soil "activation" of the chemical, can now be tentatively defined by the following pathway scheme:



Rapid breakdown of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to 2-(2,4-dichlorophenoxy)ethanol was demonstrated in soils of pH 6.0 to 7.0 and in cell-free filtrates of synthetic media cultures of *B. cereus* var. *mycoides* at pH 7.0 which would preclude acid hydrolysis. Inactivation of filtrates of *B. cereus* var. *mycoides* at temperatures (72° C.) which would cause enzyme inactivation, leads to the conclusion that the conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to 2-(2,4-dichlorophenoxy)ethanol is dependent upon enzymatic hydrolysis. Arylsulfatase activity could not be demonstrated by means of the potassium phenolphthalein disulfate reaction. However, negative results in this test do not necessarily

prove that sulfatases capable of hydrolyzing sodium 2-(2,4-dichlorophenoxy)ethyl sulfate were not present (25). No effect was noted upon 2-(2,4-dichlorophenoxy)ethyl benzoate by *B. cereus* var. *mycoides* under sterile conditions, indicating a degree of specificity of the organism for the sulfate portion of the sodium 2-(2,4-dichlorophenoxy)ethyl sulfate molecule. The qualitative detection of 2,4-D in soils treated with 2-(2,4-dichlorophenoxy)ethanol suggests a further step in the transformation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to active substances of the hormone type. 2-(2,4-Dichlorophenoxy)ethanol was more effective in inducing hormonal responses in tomato plants when the chemical was applied to soil (Table II). Foliar applications of the compound have been found less effective in inducing responses (7). Carroll (7) suggested the possibility that this was due to more rapid movement of the chemical upward in the xylem and a retarded transport downward in the phloem. It is also likely, however, that microbial conversion of 2-(2,4-dichlorophenoxy)ethanol to 2,4-D results in more pronounced hormonal effects upon plants treated in soil.

Although *Acetobacter aceti* did not oxidize the ethanol portion of the 2-(2,4-dichlorophenoxy)ethanol molecule to the acetic acid radical, a soil flora capable of performing this reaction seems necessary before 2,4-D may be formed. 2,4-D could only be detected in nonsterile soils treated with 2-(2,4-dichlorophenoxy)ethanol. The isolation of soil organisms capable of acting upon the ethanol side chain will provide an interesting addition to the array of functions assigned to soil microbes.

The pathway of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate breakdown in soil by no means ends with 2,4-D. Although this study did not progress beyond the formation of 2,4-D, other workers have recently suggested 2,4-dichlorophenol as a breakdown product of 2,4-D in soil (2). Specific soil bacteria have been shown to account for this action. It becomes increasingly evident that the application of herbicides such as sodium 2-(2,4-dichlorophenoxy)ethyl sulfate or 2,4-D to soil results in a rapid series of chemical activities by specific soil microorganisms. These activities have, in the past, been almost entirely ignored. Future research in this field offers fruitful opportunity for both the plant physiologist and soil microbiologist.

SUMMARY

The conversion of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to active plant growth regulators occurred rapidly under soil conditions which precluded acid hydrolysis. 2-(2,4-Dichlorophenoxy)ethanol, one of the hydrolysis products of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, was active in suppressing growth of cucumber roots and in modifying the growth of tomato plants. Its activity is enhanced in the presence of soil.

The oxidation of 2-(2,4-dichlorophenoxy)ethanol to 2,4-D in soil may account for this increased activity.

Bacillus cereus var. *mycoides*, isolated from soils treated with sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, converted sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to 2-(2,4-dichlorophenoxy)ethanol in neutral to alkaline soils, but did not act on 2-(2,4-dichlorophenoxy)ethyl benzoate or 2-(2,4-dichlorophenoxy)ethanol. Although arylsulfatase activity was suggested by these observations, this enzyme could not be detected by techniques employing potassium phenolphthalein disulfate.

It is believed that the herbicidal activity of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, previously referred to as soil "activation," can now be attributed to a reaction pathway involving either acid or enzymatic hydrolysis to 2-(2,4-dichlorophenoxy)ethanol, followed by oxidation of the latter compound to 2,4-D. Soil organisms converting 2-(2,4-dichlorophenoxy)ethanol to 2,4-D have not been isolated.

The present study emphasizes the need for further research involving the role of soil microorganisms in relation to the application of plant growth regulators.

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QUANTITATIVE STUDIES ON THE ROLE OF HYDROGEN SULFIDE FORMATION IN THE TOXIC ACTION OF SULFUR TO FUNGUS SPORES¹

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Although elemental sulfur has been extensively used for many years as a fungicide the mechanism of its action is little understood. Since sulfur, as such, seems rather innocuous, attempts at explanations of its mode of action have been mostly concerned with either oxidation or reduction products derived from it. Perhaps most emphasis has been placed on hydrogen sulfide as the product of the interaction between fungus spores and sulfur responsible for its toxic effect.

In the present investigations, quantitative studies have been made on the rate of hydrogen sulfide production and the total quantity produced before ability to germinate was lost for spores of nine species and two strains of fungi exposed to elemental sulfur. The relative toxicity of elemental sulfur and hydrogen sulfide has been redetermined under conditions giving more accurate control than in previous work (15) of the quantity of hydrogen sulfide available to the spores. It was found that very large amounts of hydrogen sulfide, 10,000 to 60,000 parts per million on a spore weight basis, could be formed by spores of some fungi without complete elimination of germination capacity. Since sulfur is readily oxidized and reduced in the presence of fungus spores, it is difficult to obtain an unequivocal answer as to the relative toxicity of elemental sulfur, hydrogen sulfide, and some of its oxidation products. The results indicate, however, that finely divided sulfur may be more toxic than the same quantity of hydrogen sulfide for spores of some species. It would appear, therefore, that the role of hydrogen sulfide as such in the toxic action of sulfur may not be as important as previously thought (15). It is apparent, however, that the hydrogenation of such large amounts of sulfur on a spore weight basis must seriously interfere with the necessary hydrogenation and dehydrogenation processes in the spores. The production of hydrogen sulfide may, therefore, bring about loss of viability in the spores because of this interference in the normal metabolism of the cells. Some of these results have appeared previously in abstract or summary form (12, 17, 18).

MATERIALS AND METHODS FUNGUS SPORES

Conidia from the following species of fungi were used: *Monilinia fructicola* (Wint.) Honey (4, 22) (formerly called *Sclerotinia fructicola*),

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Alternaria oleracea Milbraith, *Stemphylium sarcinaeforme* (Cav.) Wilts. (11), *Glomerella cingulata* (St.) Sp. & von S., *Aspergillus niger* van Tiegh, *Rhizopus nigricans* Ehr., *Cephalosporium acremonium* Corda, *Neurospora sitophila* (Mont.) Shear & Dodge, and *Neurospora crassa* Shear & Dodge.²

Most of the species were cultured on potato dextrose agar slants in 25-ml. or 100-ml. tubes. However, the two species of *Neurospora* were cultured in widemouthed bottles of 200-ml. capacity (16) on the medium recommended by Horowitz (6) for mutants of *Neurospora crassa* except that the DL-methionine was omitted and 30 g. of agar used per liter. This medium gave a greater yield of spores of *Neurospora sitophila* than did potato dextrose agar or modified Fries medium (20). Spores were used when the cultures were from one to several weeks old excepting those of *Monilinia fruticola* which were not used when over 10 days old. Spores of the first seven species were obtained from the cultures by adding distilled water and brushing off the spores with a rubber policeman. Spores of the *Neurospora* species were obtained by the vacuum technique previously described (16). Spores of all species were separated from water by centrifugation and were then resuspended in distilled water and centrifuged out twice more.

After exposure to the fungicide the spores were diluted to a final concentration of 100,000 per ml. and set up to germinate in drops on glass slides according to the customary slide-germination test (1, 2). Supplementary nutrients were added at the following final concentrations in grams per liter: *Monilinia fruticola*, dextrose 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0246 (Lin, 9); *Glomerella cingulata*, dextrose 0.1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.246, KNO_3 0.101, KH_2PO_4 0.136 (Lin, 10). Ultrafiltered orange juice at a concentration of 0.1 per cent was used for *Alternaria oleracea* and the Horowitz medium was used for the *Neurospora crassa* mutants. No nutrient was added to *Stemphylium sarcinaeforme*. Fries medium as modified according to Ryan, Beadle and Tatum (20, p. 785) was used for all other species.

Spores were germinated at 22° C. for 24 hours, excepting those of the species of *Neurospora* which were held at 15° or 18° C. after which germination counts were made. Untreated spores of most of the fungi gave a germination percentage of at least 95; however, the spores of *Aspergillus niger*, *Rhizopus nigricans*, and some of the *Neurospora crassa* mutants varied considerably and averaged about 70 per cent. The data for all species were adjusted to the basis of viable spores in the controls by dividing the observed germination by the per cent germination of the controls.

² Strain 16 kindly furnished by Dr. B. O. Dodge, New York Botanical Garden, New York 58, N. Y. Several mutants of *N. crassa* were also made available through the courtesy of Dr. N. H. Horowitz, California Institute of Technology, Pasadena, Calif.

DETERMINATION OF MEAN SPORE WEIGHT

The spore size was determined by measuring 50, 100, or 300 spores for the different species of fungi. Previously published data have been used in some cases (14, 15). The length and width of spores in water, chosen at random, were measured with an ocular micrometer at a magnification of 1450 \times . The spores of all species were considered to be prolate spheroids in shape excepting those of *Alternaria oleracea* which were considered a frustrum of a cone with hemispherical ends and those of *Glomerella cingulata* a cylinder with hemispherical ends. By means of the appropriate formulae the surface areas and volumes were calculated. At first the volumes were calculated on the basis of the spore of mean linear dimensions. These data were used in the earlier work. Later more accurate values were obtained by a calculation of mean volumes and mean surface areas as determined from the volumes and surface areas of individual spores. This resulted in an increase in the calculated volume of from about 3 to 10 per cent for the different species, exclusive of *Neurospora sitophila*. Because of the large variation in the size of the spores of this species, the increase in volume was about 20 per cent. Density of spores was determined by their rate of fall in water, following the original methods of Buller (3) and those reported earlier (15). This method is based on Stokes' law governing the rate of fall of spherical bodies. The spores of many of these species do not differ markedly from spheres and the radius was determined for that of a theoretical sphere equal in volume to the spore of mean volume. Mean densities so obtained were as follows: *M. fructicola* 1.07 originally (15), 1.06 in a redetermination, *G. cingulata* 1.08, *A. oleracea* 1.10, *N. sitophila* 1.11, and *S. sarcinaeforme* 1.14. Insofar as these densities varied but little and such variation was within the error of determining the spore concentrations, the remaining species were assumed to have a density of 1.1. The weights of the mean spores as thus finally calculated are given in Table I together with the mean linear dimensions, surface areas, and volumes. The dimensions for the spores of *Rhizopus nigricans* are considerably smaller than is typical. This isolate was obtained from Dr. A. F. Blakeslee in 1939 (14). In recent correspondence Dr. Blakeslee suggests that continued culture on potato dextrose agar may have brought about the reduction in size of these spores.

The spore concentrations were determined by counting appropriate dilutions in a Fuchs-Rosenthal counting cell as has customarily been done (1). At least four separate samples were counted. The agreement was reasonable for most species; however, results were sometimes erratic with *S. sarcinaeforme* due to the large size of the spores, and with *G. cingulata*, *A. niger*, and *R. nigricans* due to aggregation of the spores. For light colored spores such as *N. sitophila* and *M. fructicola* the determination of spore concentration could be satisfactorily simplified and accelerated by

the use of a photoelectric colorimeter (23). However, the use of a colorimeter for dark spores or those that liberated a dark pigment such as *Aspergillus niger* was not satisfactory.

TABLE I
MEAN SPORE DIMENSIONS, AREA, VOLUME, AND WEIGHT

Species	Major and minor axes, microns	Values for 1,000,000 spores		
		Surface area, cm. ²	Vol., mm. ³	Weight, mg.
<i>Stemphylium sarcinaeforme</i>	28.2, 23.5	20.11	8.68	9.90
<i>Monilinia fruticola</i>	15.0, 9.5	4.03	0.742	0.796
<i>Alternaria oleracea</i>	18.4, 8.2, 5.1	4.00	0.653	0.718
<i>Neurospora sitophila</i>	8.8, 7.4	2.03	0.313	0.347
<i>Glomerella cingulata</i>	15.5, 4.9	2.37	0.262	0.283
<i>Neurospora crassa</i>	6.4, 4.9	0.929	0.0863	0.0949
<i>Rhizopus nigricans</i>	5.1, 4.2	0.634	0.0519	0.0571
<i>Aspergillus niger</i>	3.8, 3.4	0.400	0.0247	0.0272
<i>Cephalosporium acremonium</i>	3.5, 1.6	0.151	0.0050	0.0055

FORMS OF SULFUR USED

Most of the work with sulfur was carried out by conventional chemical methods. Some use was made, however, of radioactive sulfur S³⁵ to check some of the conclusions reached.³ For this purpose elemental sulfur of high specific activity was available.

It was found that, in the tests in which the uptake of sulfur and the release of hydrogen sulfide were determined quantitatively, the physical state of the sulfur was extremely important. Experiments were carried out with ordinary finely ground sulfur and with various commercial formulations such as Mike sulfur⁴ and Magnetic sulfur.⁴ The form of sulfur most rapidly taken up and most toxic was a colloidal preparation obtained by dissolving sulfur in acetone and then adding the acetone solution to water. This produced a milky suspension which was relatively stable for several days. Usually 125 mg. of sulfur was dissolved in 250 ml. of acetone and several ml. of this solution, the exact amount depending upon the quantity of sulfur desired, added to 50 ml. of a suspension of fungus spores in phosphate buffer at pH 7.0. A small amount of acetone was thus present in the suspension but tests showed that the quantities used did not affect the germinability of the spores.

³ Elemental S³⁵ was obtained from the United States Atomic Energy Commission, Oak Ridge, Tenn.

⁴ Product of Dow Chemical Company, Midland, Michigan, and Stauffer Chemical Company, New York, N. Y., respectively.

DETERMINATION OF THE SULFUR ABSORBED AND
HYDROGEN SULFIDE PRODUCED

When spores of most species of fungi are suspended in aqueous media containing elemental sulfur, production of hydrogen sulfide begins almost immediately. The interaction between spores and elemental sulfur was studied by determining quantitatively the rate at which hydrogen sulfide production took place with spores of various species and by determining the total amount produced before failure of germination capacity occurred. It was found that essentially all the sulfur taken in was again given off as hydrogen sulfide, and that therefore a measure of hydrogen sulfide production was a measure of sulfur uptake.

A known quantity of freshly-collected spores, varying from a few milligrams to several hundred milligrams, depending upon the nature of the particular experiment, was suspended in 50 ml. of phosphate buffer at pH 7.0. The desired quantity of sulfur was then added and nitrogen slowly bubbled through the spore suspension and then through small tubes containing 2 to 3 ml. of a 2 per cent solution of zinc acetate. The presence of sulfide was indicated by the appearance of a white precipitate of zinc sulfide. At suitable intervals, the tubes were disconnected temporarily, fresh tubes containing zinc acetate inserted, and the amount of zinc sulfide determined iodimetrically. The zinc sulfide was dissolved by adding a small quantity of dilute hydrochloric acid and the amount of hydrogen sulfide determined by adding an excess of 0.1 *N* or 0.01 *N* iodine followed by back titration with standard sodium thiosulfate using starch as an indicator. By this method quantities of hydrogen sulfide as low as 0.02 mg. could be determined accurately. In the tests in which a small amount of acetone was present from the colloidal sulfur preparations used, the tubes containing the zinc sulfide precipitates were heated to drive off the acetone before the titrations were made.

Effect on germination was followed by taking a small quantity of spores at each sampling period and determining the percentage of germination as described above.

Comparative activity of various species was determined by noting the rate of hydrogen sulfide production when similar quantities of spores and sulfur of the same physical form were brought together. The amount of hydrogen sulfide given off, and therefore sulfur absorbed before the spores lost their ability to germinate, was determined by setting up a series of tests in which the quantity of spores varied and an excess of sulfur was usually available. Under these conditions, hydrogen sulfide production would stop after a time because of the loss of viability of the spores and the amount of hydrogen sulfide given off per unit of spore weight was usually quite close with varying quantities of spores. This would then be a measure of the dose required to prevent germination.

DETERMINATION OF COMPARATIVE TOXICITY OF HYDROGEN
SULFIDE AND ELEMENTAL SULFUR

In addition to the experiments in which the effect of the production of hydrogen sulfide by fungus spores from sulfur on their germination capacity was studied, the direct toxic action of supplying hydrogen sulfide was also investigated. Two methods were used which, in general, gave similar results. Weighed quantities of silicon sulfide, which hydrolyzes in the presence of water to produce hydrogen sulfide, were added to freshly boiled and cooled water to which a suspension of spores was also added. The containers (5-ml. bottles) with the silicon sulfide and spores were completely filled and tightly capped for 24 hours and held at 21° C. At the end of this period suitable aliquots were taken and germination capacity determined by the slide-germination technique (1, 2). In the second method solutions of hydrogen sulfide in water were made up, their strength determined by titration with iodine, and spores exposed as indicated above. The toxicity of elemental sulfur was similarly tested on spores of the same lot by having a 24-hour period of contact in a closed container before determining germination capacity by the slide-germination test.

RESULTS

EFFECT OF PHYSICAL STATE AND FORMULATION OF SULFUR

The effect of the form of sulfur on the hydrogen sulfide produced by fungus spores is shown by the data in Table II. In the upper part of the table the amount of uptake of sulfur as shown by the release of hydrogen

TABLE II
EFFECT OF PHYSICAL STATE AND FORMULATION OF ELEMENTAL SULFUR ON
THE RATE OF HYDROGEN SULFIDE FORMATION BY FUNGUS SPORES

Form of sulfur*	Spores		Hydrogen sulfide formed		
	Species	Mg.	Time, hrs.	Mg.	Relative rate
Refined flowers	<i>Neurospora sitophila</i>	300	5.3	0.046	0.06
Anchor Brand sublimed				0.16	0.19
Mike				0.58	0.70
Magnetic				0.83	1.00
Magnetic	<i>Neurospora crassa</i>	80	2	0.094	0.11
Colloidal				0.85	1.00
Magnetic plus acetone				0.29	0.34

* Refined flowers and Anchor Brand sublimed represent two preparations of pure sulfur, Mike and Magnetic are two commercial formulations designed for use as fungicides, and the colloidal preparation was made by dissolving sulfur in acetone and adding the acetone solution to water.

sulfide is given for *Neurospora sitophila* when exposed to two samples of pure sulfur and two formulated preparations recommended for fungicidal use. The latter were taken up from 13 to 18 times as readily as the refined flowers of sulfur and from 4 to 6 times as readily as the sublimed sulfur. In another experiment in which *Neurospora crassa* was used the most active of the formulated preparations (Magnetic sulfur) was compared with colloidal sulfur made by adding an acetone solution of sulfur to water. The colloidal sulfur was nine times as effective as Magnetic sulfur. In a test in which an amount of acetone equivalent to that present in the colloidal sulfur preparations was added to Magnetic sulfur an increased effect was also obtained. It appears that the effectiveness of the colloidal sulfur used is, at least in part, the result of the somewhat greater solubility of sulfur in the acetone-water mixture than in water alone. This very large effect of the form of sulfur indicates that the limiting factor in the process being studied is the availability of the sulfur to the spores rather than the capacity of the spores to reduce sulfur to hydrogen sulfide.

EXTENT OF RECOVERY OF SULFUR AS HYDROGEN SULFIDE

In Table III are shown the data obtained from six replicate tests in which 105-mg. portions of spores of *Neurospora sitophila* were treated with 2 mg. of sulfur and the recovery of hydrogen sulfide determined. It is seen

TABLE III

DATA SHOWING COMPLETENESS OF RECOVERY AS HYDROGEN SULFIDE OF SULFUR ADDED TO SIX REPLICATE LOTS OF SPORES OF *NEUROSPORA SITOPHILA*

Hydrogen sulfide given off, mg.			Per cent of added S recovered	Dose received by spores, p.p.m.*
First 2 hrs.	Next 15.8 hrs.	Total		
1.19	0.77	1.96	92	18,660
1.05	0.88	1.93	91	18,380
0.96	0.96	1.92	90	18,290
1.22	0.74	1.96	92	18,660
1.08	0.92	2.00	94	19,050
1.07	0.92	1.99	93	18,950

* Calculated on basis of H₂S given off.

that from 90 to 94 per cent of the sulfur added was recovered and that at least half of this was obtained in the first two hours. The data also illustrate the degree of concordance between replicates that can be expected in these experiments on hydrogen sulfide production. It was concluded, in view of the instability of hydrogen sulfide and some expected loss therefore in the procedures used, that all of the sulfur taken in is again given off as hydrogen sulfide. Tests in which radioactive sulfur was used have confirmed this conclusion. The radioactivity was recovered in the hydrogen sulfide. Some slight amount of radioactivity remained in the sulfur frac-

tions in the spores but this was presumably the result of exchange between the natural sulfur compounds in the spores and the sulfur which was applied. Similarly, when spores which contained radioactive sulfur were used and the elemental sulfur applied was not radioactive, only a very small fraction of the hydrogen sulfide recovered was radioactive. This again presumably is the result of small amounts of exchange between the sulfur taken in and that already present.

The data in Table III also illustrate the rather high dose received by the spores in this test. If one considers the hydrogen sulfide produced as the dose, the spores received an average of 10,400 p.p.m. of their weight during the first two hours. At the end of 17.8 hours when the sulfur had been exhausted, they had received between 18,000 and 19,000 p.p.m. Actually the tests were continued after adding a further 0.8 mg. of sulfur and hydrogen sulfide production again was resumed and when the experiment was ended the spores had received an average total of 24,000 p.p.m. of sulfur.

RATE OF HYDROGEN SULFIDE PRODUCTION BY SPORES
OF VARIOUS SPECIES

Since spores of different species vary widely in their sensitivity to sulfur it was of interest to determine the relative capacity of various species to take up sulfur and give off hydrogen sulfide. Usually 50 to 100 mg. of spores were suspended in 50 ml. of water in which 2 to 3 mg. of sulfur in

TABLE IV
RATE OF FORMATION OF HYDROGEN SULFIDE BY FUNGUS
SPORES EXPOSED TO ELEMENTAL SULFUR

Species	Mg. H ₂ S per g. of spores per hr.	
	Mean	Standard error
<i>Cephalosporium acremonium</i>	6.39	1.35
<i>Neurospora sitophila</i>	3.39	0.20
<i>Neurospora crassa</i>	3.13	0.19
<i>Glomerella cingulata</i>	0.98	0.10
<i>Monilinia fructicola</i>	0.93	0.14
<i>Alternaria oleracea</i>	0.36	
<i>Aspergillus niger</i>	0.085	0.024
<i>Stemphylium sarcinaeforme</i>	0.074	
<i>Rhizopus nigricans</i>	0.023	

acetone were added. A stream of nitrogen was passed through and the hydrogen sulfide produced in the first two hours determined as well as that produced on further exposure. Comparisons of the rate for the first two hours were made because, with the exceptions noted below, the rate was the highest during this period. The results with nine species are summarized in Table IV. Data are given for the rate of hydrogen sulfide production expressed as mg. of hydrogen sulfide per gram of spores per hour.

(To convert to parts per million, the values, of course, have to be multiplied by 1000.) In each instance the values are calculated from the production for the first two hours. Since with many of the species figures were available for a considerable number of runs, the standard errors of the averages were calculated and are shown in column three of the table.

Spores of different species varied widely in the rate at which they gave off hydrogen sulfide after exposure to sulfur. Spores of *Cephalosporium acremonium* produced about 275 times as much hydrogen sulfide as those of *Rhizopus nigricans* during the first two hours and nearly twice as much as those of the second most active species, *Neurospora sitophila*. Moreover, *C. acremonium* is one of the species characterized by production at a greater rate during the second two-hour period than the first so that its activity is greater in comparison to the other species than the data of Table IV indicate. Uptake during the second two-hour period for this species is usually 20 to 30 per cent higher than for the earlier period.

Among the other species listed, spores of *Aspergillus niger* have also shown a higher rate of hydrogen sulfide production for time periods subsequent to the first two hours. The rate with this species is very low but because hydrogen sulfide production continues for a considerable period of time the total amount involved may be quite large. In one experiment in which the rate of hydrogen sulfide production was determined for a series of spore concentrations varying from 40 to 320 mg. in 50 ml. of water to which 1.8 mg. of sulfur had been added, the average per gram of spores for the first two-hour period was 0.0851 mg. per hour, for the next 17 hours, 0.1092 mg., and for the next 23.8 hours, 0.124 mg. The spores, therefore, released an amount of hydrogen sulfide equivalent to from 0.41 to 0.56 per cent of spore weight, or 4100 to 5600 p.p.m. There was also some indication that the other low producers of hydrogen sulfide, namely *Stemphylium sarcinaeforme* and *Rhizopus nigricans*, may continue after two hours at a rate substantially the same or even a little higher.

Resistance of the Spores of Some Species to Penetration of Sulfur

Slow rate of hydrogen sulfide production by some species of spores could be the result of reduced capacity to form hydrogen sulfide from sulfur or lessened capacity to absorb sulfur or a combination of both these factors. The results show that when little hydrogen sulfide is recovered, the sulfur remains substantially outside the fungal cells. In an experiment in which 1 mg. of sulfur was added to spores of *Rhizopus nigricans* and only 0.077 and 0.063 mg. of hydrogen sulfide were recovered after 18.5 and a further 23.5 hours respectively, an additional 0.77 mg. of H_2S was obtained after spores of *Neurospora sitophila* were added. As a matter of fact, since the colloidal sulfur imparted a milkyiness to the solutions it was always possible to observe by simple inspection whether appreciable quantities of sulfur

remained unabsorbed. Because of the great activity of spores of such species as *Neurospora sitophila* (yeast cells could also be used) in giving quantitative recovery of sulfur as hydrogen sulfide, exact determinations of the unabsorbed sulfur could be made when required.

QUANTITIES OF SULFUR REQUIRED TO RENDER SPORES
INCAPABLE OF GERMINATION

Since determinations of germination capacity were also made during the course of the studies on rate of hydrogen sulfide production, much information was obtained on the effects of the uptake of sulfur on the viability of spores of a number of species. The results of various tests have indicated that the production of hydrogen sulfide stops when spores lose their ability to germinate. This conclusion is based on the following: (a) hydrogen sulfide production does not occur when mechanically ground preparations of spores are mixed with elemental sulfur; (b) when various materials known to be fungicidal are added to spores at the same time as elemental sulfur, there is either no production of hydrogen sulfide or it ceases early, apparently as soon as the other toxicant has had an opportunity to exert its action; (c) when various quantities of spores are exposed to sulfur in excess, hydrogen sulfide production stops when the spores have given off approximately the same amount of hydrogen sulfide on a spore weight basis.

Data from experiments in which the quantity of spores was varied over a fairly wide range are given in Table V and the differing sensitivity of spores of various species is indicated. The species are listed in the same order as in the preceding table, based on the relative rates of hydrogen sulfide production. Values for *Neurospora sitophila* are not included in the table but are given in Figure 1 in the form of a dosage-response curve. The data show that the species most sensitive to sulfur are in an intermediate position in regard to the rapidity with which they reduce sulfur to hydrogen sulfide. Some of the species which take up sulfur most rapidly, such as *Cephalosporium acremonium*, *Neurospora sitophila*, *Neurospora crassa* and two of its mutants² require from 10,000 to over 50,000 p.p.m. on a spore weight basis before serious interference with or loss of germination capacity becomes evident. Especially resistant are the spores of *C. acremonium* which are the most active in absorbing sulfur and forming hydrogen sulfide of all the species tested. Most sensitive are spores of *Glomerella cingulata*, *Monilinia fructicola*, and *Alternaria oleracea* which are completely inhibited in germination after about 4500 to 8800 p.p.m. of hydrogen sulfide have been given off. In a separate class are spores of *Aspergillus niger*, *Stemphylium sarcinaeforme*, and *Rhizopus nigricans* which take up sulfur only slowly and the germination capacity of which was little affected under the conditions of the tests. Values for a number of experiments with *Neurospora*

TABLE V
PRODUCTION OF HYDROGEN SULFIDE AND SURVIVAL OF SPORES
EXPOSED TO ELEMENTAL SULFUR

Species	Wt. of spores, mg.	H ₂ S produced			Germination, %
		Time, hours	Mg.	P.p.m. of spore wt.	
<i>Cephalosporium acremonium</i>	50	117.8	2.68	53,600	28
	81		3.18	39,750	4
<i>Neurospora crassa</i>	23	23.0	0.523	22,700	4
	45		0.984	21,900	4
	90		1.840	20,400	31
<i>Neurospora crassa</i> mutant	40	28.7	0.447	11,200	0
	80		0.970	12,100	0
	80		0.773	9,700	0
<i>Neurospora crassa</i> mutant	37	18.9	0.647	11,400	4
	114		1.283	11,300	2
	114		1.464	12,800	0
<i>Glomerella cingulata</i>	40	22.7	0.26	6,500	0
	80		0.61	7,600	0
	160		1.41	8,800	0
	320		1.73*	5,400*	0
	640		1.77*	2,800*	0
<i>Monilinia fructicola</i>	40	26.8	0.28	6,900	0
	80		0.35	4,400	0
	160		0.74	4,600	0
	320		1.57	4,900	0
<i>Alternaria oleracea</i>	40	53.6	0.315	7,900	4
	80		0.646	8,100	6
	160		1.023	6,400	4
	320		1.484*	4,600*	6
<i>Aspergillus niger</i>	40	42.8	0.225	5,600	97
	80		0.414	5,200	91
	160		0.800	5,000	87
	320		1.300	4,100	74
<i>Stemphylium sarcinaeforme</i>	94	20	0.0409	430	91
	188		0.0221	120	71
	376		0.0340	90	84
	752		0.0442	60	92
<i>Rhizopus nigricans</i>	65	19.5	0.275	9,700	96

* Quantity of sulfur available was limiting in these tests.

sitophila summarized in the curve of Figure 1 indicate an ED₅₀ value of about 11,500 p.p.m. for this species.

It should be pointed out that these tests were made under anaerobic conditions and that the relative sensitivity of the spores of the various species is not necessarily the same when the slide-germination technique is used. Spores of *Alternaria oleracea*, for example, are relatively more resist-

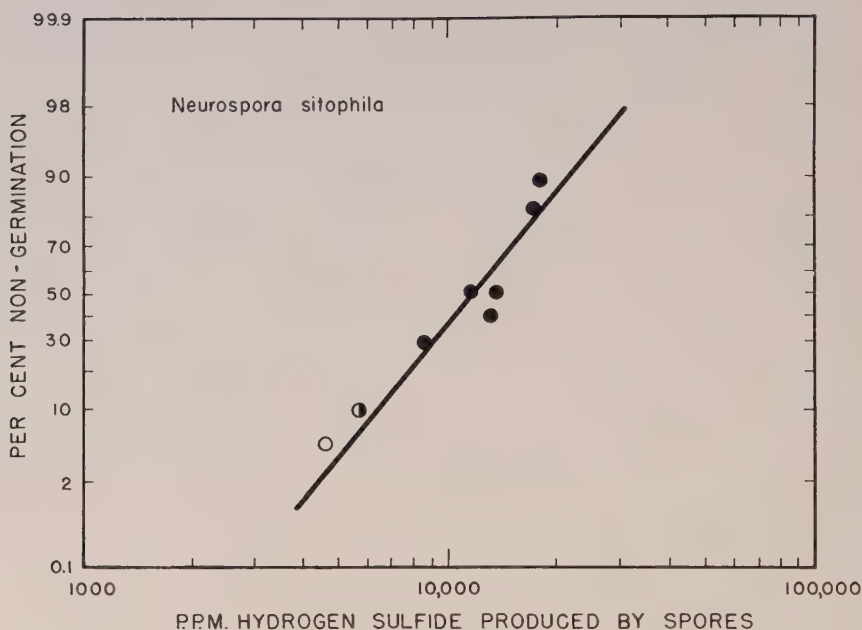


FIGURE 1. Dosage-response curve for the action of sulfur on the spores of *Neurospora sitophila* as measured by the production of hydrogen sulfide on a spore weight basis. Different kinds of circles indicate different experiments.

ant to sulfur when tested on slides than in the experiments reported here. Preliminary experiments using S^{35} have shown that when the spore-sulfur suspension is allowed to stand in air some of the sulfur is oxidized to sulfate. Differences in the behavior of the sulfur, therefore, probably play a role in sensitivity of spores under aerobic conditions as compared to anaerobic conditions.

COMPARATIVE TOXICITY OF HYDROGEN SULFIDE AND COLLOIDAL SULFUR

Studies were carried out on the effect of hydrogen sulfide on the germination capacity when supplied in aqueous solutions to suspensions of spores of a number of species. Because of the readiness with which hydrogen sulfide escapes into the surrounding atmosphere and also because of its instability in the presence of oxygen, the spores were exposed in completely filled, closed containers for 24 hours as explained in the Materials and Methods section. The data are presented in Table VI. Data for colloidal sulfur and for a wettable sulfur (Magnetic sulfur) are also included. The tests with colloidal sulfur and wettable sulfur were conducted under the same conditions as those with the hydrogen sulfide solutions.

The data show that hydrogen sulfide was significantly less toxic than colloidal sulfur to spores of five of the eight species. With the other three the differences were not significant. With Magnetic sulfur an ED₅₀ value could be obtained only for *Monilinia fructicola*. While it is quite likely that there was some destruction of hydrogen sulfide during the course of the tests the differences in toxicity, based on the concentration of the external solutions, are so large as to indicate quite clearly a higher toxicity for

TABLE VI
ED₅₀ VALUES IN P.P.M. OF EXTERNAL SUSPENSION OR SOLUTION, FOLLOWING
24-HOUR FUNGICIDAL EXPOSURE TO WETTABLE SULFUR, COLLOIDAL
SULFUR, AND HYDROGEN SULFIDE (MEAN OF 2 TO 4 TESTS)

Species	Wettable sulfur	Colloidal sulfur	Hydrogen sulfide
<i>Monilinia fructicola</i>	54	0.5	2.8**
<i>Cephalosporium acremonium</i>	> 1000	0.3	12**
<i>Aspergillus niger</i>	> 1000	0.3	15**
<i>Glomerella cingulata</i>	> 1000	0.4	20**
<i>Neurospora sitophila</i>	> 1000	1.0	38**
<i>Rhizopus nigricans</i>	> 1000	2.7	5.9
<i>Alternaria oleracea</i>	> 1000	18	15
<i>Stemphylium sarcinaeforme</i>	> 1000	31	8.8

** Highly significant difference between colloidal sulfur and hydrogen sulfide.

colloidal sulfur than for an equivalent amount of hydrogen sulfide for some of the species included. The ED₅₀ values for the five species for which the differences are significant varied from 5.6 to 50 times higher for hydrogen sulfide than for colloidal sulfur. Ample allowance for the instability of hydrogen sulfide would still indicate a lower toxicity for it than for colloidal sulfur. It should be emphasized that these values are for the concentrations of the external solutions or suspensions and that the actual dose on a spore weight basis remains unknown.

STUDIES ON THE CHARACTERISTICS OF THE SULFUR REDUCING SYSTEM

Effect of Inhibitors

Some of the more common enzyme inhibitors were tested for their effect on the reduction of sulfur by spores of *Neurospora sitophila* and yeast cells to hydrogen sulfide (Table VII). The system is not sensitive to cyanide, semicarbazide, hydroxylamine, or sodium azide. Sodium bisulfite exerts considerable inhibitory effect at a concentration of 1×10^{-3} molar. The most marked inhibition is brought about by sodium arsenite. Concentrations of 1×10^{-3} molar inhibit the formation of hydrogen sulfide completely and some effect is still evident at 6.4×10^{-5} molar. Concentrations which inhibited reduction of sulfur also affected the germination capacity adversely. When arsenite prevented the formation of hydrogen sulfide, the

elemental sulfur remained in solution. This could be demonstrated by adding a fresh lot of spores or yeast cells after a time. Apparently the arsenite was no longer present as such and the sulfur could now be recovered as hydrogen sulfide. The spores originally present were, however, no longer viable and were therefore unable to reduce the sulfur.

Influence of sodium arsenite on toxicity of colloidal sulfur. This effect of sodium arsenite in preventing the reduction of sulfur seemed to offer a

TABLE VII
EFFECT OF SOME ENZYME INHIBITORS ON THE REDUCTION OF SULFUR TO HYDROGEN SULFIDE BY SPORES OF *NEUROSPORA SITOPHILA* AND YEAST CELLS

Inhibitor	Molar concn.	Per cent inhibition
Yeast cells		
Potassium cyanide	1×10^{-3}	17
Semicarbazide		14
Sodium azide		7
Hydroxylamine		11
Sodium bisulfite		71
Sodium arsenite		100
Spores of <i>Neurospora sitophila</i>		
Potassium cyanide	4×10^{-3}	39
Semicarbazide	4×10^{-3}	2
Hydroxylamine	4×10^{-3}	4
Sodium bisulfite	4×10^{-3}	65
Sodium bisulfite	1×10^{-3}	20
Sodium bisulfite	2.5×10^{-4}	16
Sodium arsenite	1×10^{-3}	100
Sodium arsenite	5×10^{-4}	97
Sodium arsenite	2.5×10^{-4}	89
Sodium arsenite	6.4×10^{-5}	21
Sodium arsenite	4.0×10^{-6}	0

means of studying the toxic action of sulfur under conditions in which reduction to hydrogen sulfide did not take place (12). The ED₅₀ values for colloidal sulfur and for colloidal sulfur plus a concentration of sodium arsenite known to prevent hydrogen sulfide formation under anaerobic conditions were determined. These tests were carried out by the slide-germination technique under aerobic conditions in contrast to the anaerobic conditions prevailing when hydrogen sulfide was determined by passing nitrogen through the spore suspensions. The values for *N. sitophila*, *Cephalosporium acremonium*, and *Aspergillus niger* for colloidal sulfur were 1.5, 5.0, and 1.2 p.p.m. respectively, and for colloidal sulfur plus 0.002 molar sodium arsenite, 1.2, 4.5, and 1.2 p.p.m. expressed in terms of the concentration of the external solution. Sodium arsenite at 0.002 molar had no effect on germination. In contrast, spores exposed to this concentration

under anaerobic conditions failed to germinate. Using radioactive sulfur it was shown that hydrogen sulfide was given off from the drops in the slide-germination tests at concentrations which completely prevented the reduction of sulfur under ^{anaerobic} aerobic conditions. The decreased effectiveness of sodium arsenite under these conditions was probably the result of an effect on the sodium arsenite itself rather than any difference in the sensitivity to arsenite of the enzyme systems involved.

Effect of Methylene Blue

When methylene blue was added to the system, providing competition for hydrogen, results as shown in Table VIII were obtained. It seems that hydrogen was more readily transferred to methylene blue than to sulfur.

TABLE VIII
EFFECT OF METHYLENE BLUE ON THE REDUCTION OF SULFUR TO HYDROGEN
SULFIDE BY SPORES OF NEUROSPORA SITOPHILA

Methylene blue, mg. per liter	Hydrogen sulfide recovered, mg.		
	First 2.4 hrs.	Next 13.8 hrs.	Total
128	0.17	0.52	0.69
32	0.75	0.45	1.20
8	0.92	0.24	1.16
2	0.92	0.12	1.04
0	1.12	0.14	1.26

Although some hydrogen sulfide was given off before the methylene blue was decolorized, the bulk of the hydrogenation of sulfur took place after the methylene blue had been decolorized.

Effect of Adding Other Fungicides

A number of experiments were also run in which the effect of adding other fungicides to the mixture of colloidal sulfur and spores was studied. In general hydrogen sulfide production was less under these conditions. The time required for the production of hydrogen sulfide to cease entirely differed with the various toxicants and this was perhaps a measure of the rate at which the toxic effect was exerted on the spores. Only the data with 2,3-dichloro-1,4-naphthoquinone will be presented here since this compound was active at low concentrations in preventing the recovery of any hydrogen sulfide from the sulfur present. The data are given in Table IX. The naphthoquinone was as effective, if not more effective, than sodium arsenite in inhibiting the formation of hydrogen sulfide. Whether the naphthoquinone or sodium arsenite has a direct effect on the enzyme systems involved or whether they exert their influence indirectly is not clear from the data at hand.

AEROBIC VERSUS ANAEROBIC CONDITIONS

These tests on the formation of hydrogen sulfide were all carried out under anaerobic conditions in that nitrogen was passed through the spore suspensions in order to drive off the hydrogen sulfide for absorption by solutions of zinc acetate and subsequent quantitative determination. If air had been passed through, some of the hydrogen sulfide formed would have been oxidized to elemental sulfur and also some of the sulfur present would have been oxidized to various other products. Under the usual conditions when sulfur is applied for the purpose of controlling fungus diseases aerobic conditions prevail. Preliminary experiments in which air or carbon dioxide was used as the flushing gas instead of nitrogen, have not indicated any marked difference in the effect on the germination capacity of the spores concerned. When spores were allowed to stand in suspension with sulfur

TABLE IX
INHIBITION OF REDUCTION OF SULFUR BY FUNGUS SPORES TO HYDROGEN
SULFIDE BY 2,3-DICHLORO-1,4-NAPHTHOQUINONE

Molar concn.	Per cent inhibition	Molar concn.	Per cent inhibition
6.0×10^{-4}	100	4.4×10^{-5}	55
1.1×10^{-4}	89	2.2×10^{-5}	51
8.8×10^{-5}	80	4.4×10^{-6}	0

without any gas being passed through, some of the sulfur was oxidized to sulfate. This was demonstrated by the use of S^{35} . Samples of spores obtained after such treatment from the bottom of the tubes in which the experiments were carried out included a certain amount of radioactivity, presumably due to elemental sulfur, which could be driven off by exposure under a heat lamp. The S^{35} which could not be driven off under the heat lamp was found to be in the form of the sulfate ion.

DISCUSSION

The fact that many kinds of living tissue can reduce sulfur to hydrogen sulfide has been known for a long time. This reaction was studied extensively by De Rey-Pailhade (19) in 1888 and he probably was dealing with glutathione at that time. It remained for Hopkins (5) to isolate and name glutathione from yeast and liver many years later. Glutathione will react with elemental sulfur at room temperature to give hydrogen sulfide and be itself oxidized. Since glutathione is widely distributed in living tissue, the formation of small amounts of hydrogen sulfide after the addition of sulfur to such tissue can easily be demonstrated. However, the production of hydrogen sulfide from sulfur by fungus spores is not merely a simple chemical reaction between sulfur and a sulfhydryl compound. This was recog-

nized in earlier work (15) in which the enzymic nature of the process was indicated and in which it was pointed out that it was unlikely that fungus spores contained enough of a sulfhydryl compound to account for all the hydrogen sulfide that could be given off. Actually, it was found in the present investigations that the rate of hydrogen sulfide formation on the addition of elemental sulfur to fungus spores was much higher than when glutathione and sulfur were allowed to react directly under comparable conditions. This was true, even though with the spores, there was the question of bringing sulfur into contact with the proper reactants while with the chemical system direct contact was immediately established. If glutathione plays a role in the production of hydrogen sulfide by fungus spores an enzyme must play an active part in catalyzing the relatively slow reaction between sulfur and glutathione. Similarly, since the quantities involved are so large, it would seem that a second enzyme system must be necessary to reduce the oxidized glutathione to make it available again for the reduction of more sulfur.

Another possible explanation of the formation of hydrogen sulfide is that, in view of the similarity between oxygen and sulfur, hydrogen is transferred to sulfur instead of oxygen in the terminal oxidase system involved in respiration. This remains a possibility perhaps, but it is significant that the reduction of sulfur is not inhibited by 1×10^{-3} molar KCN, either with yeast cells or spores of *Neurospora sitophila*. Sciarini and Nord (21) studied the formation of hydrogen sulfide from elemental sulfur in the course of alcoholic fermentation by *Fusaria*. They concluded that some of the hydrogen freed by the dehydrogenases, and acceptable either by oxygen, nitrates, or some intermediates present, is diverted to elemental sulfur, which is taking a part in the competitive chain of reactions. If such is the case with fungus spores subjected to sulfur it could be that the detrimental effect of sulfur may be the result of interference with the normal metabolic processes and shifting of the originally established equilibrium between hydrogen donors and acceptors.

In the presentation of the data on the interaction between fungus spores and elemental sulfur it has been assumed that the sulfur added had to be taken in, at least by the spore membranes, before reduction to hydrogen sulfide could take place. This assumption was based on a number of experimental facts. In the first place, ground up spores when mixed with sulfur did not reduce it to hydrogen sulfide. The addition of the supernatant from spore suspensions did not have a measurable effect on the rate of hydrogen sulfide formation. When spores were suspended in water and separated by a collodion membrane from the elemental sulfur, appreciable reduction of sulfur did not occur until the membrane was broken. Previous experiments (15) have shown that when a spore suspension was separated from sulfur paste by a collodion membrane hydrogen sulfide could be

demonstrated only on the side containing the spores. There was no diffusion of any material from the spores into the sulfur paste which resulted in the formation of hydrogen sulfide, even though the lead acetate test method would have detected less than 0.0033 mg.

Various proposals as to the mode of action of sulfur in killing fungus spores are summarized in the book on fungicides by Horsfall (7). They are concerned either with oxidation or reduction products of sulfur. Unequivocal testing of the various theories is difficult because of the interconvertibility of many of the compounds involved. Sulfur on standing in water is oxidized to various products and is of course readily reduced by fungus spores to hydrogen sulfide. The quantity of hydrogen sulfide actually produced by the spores under aerobic conditions is difficult to determine because it is easily oxidized by atmospheric oxygen to elemental sulfur. The experiments reported in the present paper have indicated clearly that the formation of hydrogen sulfide from elemental sulfur by fungus spores is not detrimental to the germination capacity of the spores concerned until relatively large quantities are involved on a spore weight basis. The importance of the production of hydrogen sulfide in the toxic action of sulfur may not be as great as was previously suggested before the relation between the reduction of sulfur and the effect on germination capacity was as well understood. Since hydrogen sulfide is so highly toxic to warm-blooded animals there may be a temptation to overstress its toxicity to other organisms. In fumigation experiments with sulfur dioxide, chlorine, ammonia, hydrogen sulfide, and hydrogen cyanide actively growing cultures of various plant pathogens showed considerable resistance to hydrogen sulfide as compared to some of the other gases studied (13). For use as a fumigant to control seed-borne fungus diseases, concentrations ranging from 300 to 500,000 p.p.m. for exposure periods of from 48 to 168 hours have been suggested (8).

The determination of the toxicity of hydrogen sulfide to fungus spores is complicated by the fact that hydrogen sulfide is a gas readily given off into the air from aqueous solutions and also easily oxidized by molecular oxygen to elemental sulfur. If tests are carried out by the procedures usually used in the slide-germination technique hydrogen sulfide will diffuse rapidly into the surrounding air and the spores will be exposed to concentrations and actual dosages much lower than indicated by the strength of the solutions originally started with. On the other hand, attempts to correct this by maintaining a given concentration in the drops containing the spores (15) by adding the required quantities of hydrogen sulfide to the air in the container as determined by Henry's law have the effect of making available an extremely large reservoir of hydrogen sulfide on a spore weight basis. The methods used in the present experiments, while not without some disadvantages, avoid these extremes.

These experiments suggest that the role of hydrogen sulfide as such may have been previously exaggerated. Until further evidence is available to give a more concrete explanation it would appear that interference with the normal dehydrogenation and hydrogenation reactions may be of more importance in explaining the toxicity of sulfur than the direct toxic effect of hydrogen sulfide.

Determination of the rate of hydrogen sulfide formation on exposure to sulfur brings out the extremely large effect of formulation. The results suggest that a good laboratory test for the efficacy of various formulations would be the rate of penetration of sulfur as shown by the hydrogen sulfide production. The greatly increased efficacy of the colloidal sulfur used in the experiments reported here indicates that much improved formulations for commercial use are possible.

SUMMARY

When spores of many species of fungi are brought in contact with elemental sulfur prompt evolution of hydrogen sulfide takes place. If the amount of sulfur present is not excessive, quantitative recovery of the sulfur as hydrogen sulfide is obtained. The hydrogen sulfide given off can therefore be used as a measure of the quantity of sulfur taken up.

The rate at which spores could absorb sulfur and give off hydrogen sulfide was greatly influenced by the physical state and formulation of the sulfur used. Commercially formulated sulfurs were three to five times as active as the best sample of pure sulfur. A colloidal preparation made by adding a solution of sulfur in acetone to water was taken up about nine times as readily as the most active commercially formulated sample tested. The results suggest that determination of the rate of hydrogen sulfide production is a good test of the efficacy of a formulation. The greatly improved performance of the colloidal sulfur preparation also indicates that more efficacious formulations for commercial use are possible.

The rate of production of hydrogen sulfide from colloidal sulfur and the total amount produced before inability to germinate occurred, was determined for the spores of nine species and two strains of fungi. The rate of production expressed as milligrams of hydrogen sulfide per gram of spores per hour varied from 0.023 for *Rhizopus nigricans* to 6.39 for *Cephalosporium acremonium*. The total amount taken up before inability to germinate occurred, varied from about 4500 to 10,000 p.p.m. on a spore weight basis for *Alternaria oleracea*, *Monilinia fruticola*, *Glomerella cingulata*, and mutants of *Neurospora crassa*. *Neurospora crassa* gave off up to 22,000 p.p.m. and *Cephalosporium acremonium* up to 54,000 p.p.m. without complete inhibition of germination. The ED₅₀ value for *Neurospora sitophila* based on hydrogen sulfide given off was about 11,500 p.p.m. *Stemphylium sarcinaeforme* gave off up to 430 p.p.m. without affecting germination and

Aspergillus niger and *Rhizopus nigricans* germinated over 90 per cent after having given off 4000 to 9700 p.p.m. of hydrogen sulfide. Hydrogen sulfide is therefore not very toxic to fungus spores on a spore weight basis under the conditions of these tests.

Comparative tests on the toxicity of hydrogen sulfide and elemental sulfur when carried out under conditions preventing loss of hydrogen sulfide through diffusion into the atmosphere and minimizing loss through oxidation to sulfur, have shown colloidal sulfur to be more toxic than an equivalent amount of hydrogen sulfide for some fungi. In these tests concentrations were expressed on the basis of the applied solutions and the quantities taken up by the spores are not known.

The reduction of sulfur to hydrogen sulfide was not inhibited by 1×10^{-3} molar potassium cyanide, semicarbazide, hydroxylamine, or sodium azide. The system was somewhat sensitive to sodium bisulfite and especially so to sodium arsenite, which has some inhibitory effect at concentrations as low as 6.4×10^{-5} molar. The presence of another hydrogen acceptor, such as methylene blue, retarded the production of hydrogen sulfide until the methylene blue was decolorized. The addition of other fungicides in general brought about a reduction of the amount of hydrogen sulfide produced in that after a time no more reduction of sulfur took place. 2,3-Dichloro-1,4-naphthoquinone was especially active in this respect preventing the formation of hydrogen sulfide at fairly low concentrations (6.0×10^{-4}).

These results indicate that the role of hydrogen sulfide in the toxic action of sulfur to fungus spores may have been previously exaggerated. It is suggested, until a more concrete explanation becomes available, that sulfur may exert its effect through its action as a hydrogen acceptor and therefore its interference in the normal dehydrogenation and hydrogenation reactions.

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RATE OF UPTAKE AND TOXIC DOSE ON A SPORE WEIGHT BASIS OF VARIOUS FUNGICIDES¹

LAWRENCE P. MILLER, S. E. A. MCCALLAN, AND RICHARD M. WEED

In studying the toxicity of chemicals to various organisms it is customary to express the effects on the basis of the quantity of chemical required per unit weight of organism. With very small organisms such as bacteria or fungus spores, it becomes difficult to do this, and the concentration of the external solution has been accepted as the unit to be used to express toxicity. The validity of using this concentration as a basis of comparing the innate toxicities of widely different chemicals becomes impaired if there are marked differences in the dosages received by the test organisms when subjected to the same external concentrations.

With the availability of radioactive isotopes which permit the quantitative determination of very small quantities of chemicals it is possible to determine actual doses for such small organisms as bacteria and fungi. Experiments carried out with labeled 2-heptadecyl-2-imidazoline (13), silver, and cerium, and, to a lesser extent, with ferric dimethyldithiocarbamate have shown that the actual dose received by the spores may be far different, by as much as 10,000-fold or more, from that indicated by the concentration of the applied solution. Moreover, this dose may be achieved very rapidly. Spores of different species may vary widely in their affinity for various fungicides. In general, with the fungicides tested, large doses on a spore weight basis are required to inhibit germination. Some of these results have been published previously in abstract form (7, 8, 9).

MATERIALS AND METHODS

FUNGUS SPORES

Conidia from the following species of fungi were used: *Monilinia fructicola* (Wint.) Honey (formerly called *Sclerotinia fructicola*), *Alternaria oleracea* Milbraith, *Stemphylium sarcinaeforme* (Cav.) Wilts., *Glomerella cingulata* (St.) Sp. & von S., *Aspergillus niger* van Tiegh, *Rhizopus nigricans* Ehr., *Cephalosporium acremonium* Corda, *Neurospora sitophila* (Mont.) Shear & Dodge, *Venturia inaequalis* (Cke.) Wint., and *Venturia pyrina* Aderh.

Methods employed for culturing the fungi and harvesting the spores and details as to the nutrients added for germination tests are given in the preceding paper (10) except for the species of *Venturia*. Spores of *Venturia* were obtained from naturally infected fruit or leaves, from which they were

¹ These investigations were conducted in cooperation with the United States Atomic Energy Commission, Contract AT(30-1)-788.

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removed with a wet camel's hair brush. They were then separated from the water by centrifugation and were resuspended in distilled water and centrifuged out twice more. Spores of *Venturia inaequalis* were germinated in a medium containing 2 grams of sucrose and 0.01 gram of sodium citrate per liter (Miller, 6), while Fries medium as modified by Ryan, Beadle, and Tatum (12, p. 785) was employed for *Venturia pyrina*.

Spore weights and surface areas used for the calculations of dose were those given in Table I of the preceding paper. Data, similarly obtained, for the *Venturia* species are as follows for *V. inaequalis* and *V. pyrina*, respectively: major and minor axes, 19.3, 7.7, and 21.7, 6.8 microns; surface area for 10^6 spores, 3.89 and 3.88 cm^2 ; volume, 6.16 and 5.50 mm^3 ; weight, 0.690 and 0.605 mg. Spore concentrations were determined either by counting in a Fuchs-Rosenthal counting cell or by using a photoelectric colorimeter as described previously (10).

LABELED FUNGICIDES

2-Heptadecyl-2-imidazoline. Labeled 2-heptadecyl-2-imidazoline was prepared from ethylene diamine and stearic acid² with a C^{14} carbon in the carboxyl group. The resulting compound therefore had the labeled carbon in the 2 position in the ring. The preparation had a specific activity of 3.20 μc . per milligram. Because of the high specific activity it was easily possible to determine the radioactivity of microgram quantities by direct counting with no significant interference from self-absorption. Since fungus spores are small, it was also possible to determine the radioactivity of spores which had taken up the imidazoline by direct counting. Stock solutions of the labeled imidazoline containing from 10 to 30 mg. per 100 ml. in acetone were prepared. For the work with spores, 0.2-ml. portions were added to 10 ml. of a spore suspension in a 15-ml. centrifuge tube. This, then, represents concentrations of from 2 to 6 p.p.m. In this range, the imidazoline remains in solution.

Ferric dimethyldithiocarbamate. Sulfur labeled ferric dimethyldithiocarbamate³ was obtained from Tracerlab, Inc., Boston, Massachusetts. It had specific activity of 10 μc . per milligram when received.

Silver. The tests with silver were run using AgNO_3 at 1.02 p.p.m. of silver. Sufficient Ag^{110} , obtained from the Oak Ridge National Laboratory, of a specific activity of 28.1 mc. per gram, was added to the solution to give counts between 500 to 750 per minute per microgram of silver.

² The labeled stearic acid was made available from funds supplied by the Carbide and Carbon Chemicals Company, New York, N. Y. The preparation of the imidazoline was kindly carried out by Mr. Vernon Raaen of the Chemistry Division of the Oak Ridge National Laboratory, Oak Ridge, Tenn.

³ The labeled ferric dimethyldithiocarbamate was purchased with funds supplied by E. I. du Pont de Nemours Company, Inc., Wilmington, Del.

Cerium. Ce^{144} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee, carrier free as CeCl_3 . The preparations used in the experiments consisted of $\text{Ce}_2(\text{SO}_4)_3 \cdot 8\text{H}_2\text{O}$ made up to give a solution containing 10 p.p.m. of cerium ions and enough Ce^{144} to give from 300 to 600 counts per minute per 10 micrograms of cerium.

DETERMINATION OF THE UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM

For determining the rate of uptake of 2-heptadecyl-2-imidazoline, silver, and cerium, a known weight of spores was suspended in 10 ml. of an aqueous solution of the fungicide in a 15-ml. centrifuge tube with a conical bottom. At suitable time intervals the mixture was centrifuged. With most spores used, the bulk of the spores was deposited at the bottom of the tube in a few seconds, but centrifugation was usually continued for at least one minute. In time studies, the exposure of the spores to the fungicide was considered ended when centrifugation was begun and not resumed until the spores were again adequately suspended in the solution. It was thus assumed that no appreciable uptake of fungicide took place while the spores were tightly packed in the narrow conical area at the bottom of the centrifuge tubes. The amount taken up was determined by removing an aliquot of the fungicidal solution at the various time intervals and noting the loss in radioactivity. The results obtained in this manner were also checked by determining the radioactivity of an aliquot of the spores being treated. The values obtained by these two procedures agreed very well except in some experiments with the imidazoline in which the conditions of the experiment were such as to permit time for appreciable adsorption of the fungicide on the walls of the glass centrifuge tubes used. Solutions of silver and cerium could stand in glass vessels for long periods without loss of activity.

The dose received by the spores could be regulated by varying the concentration of the fungicidal solution, the quantity of spores present, or the time of exposure, or by repeated exposure to a series of solutions of the toxicant. All these methods were used. The effect on germination was brought about by the amount of fungicide taken up rather than by the method of treatment. Varying the quantity of spores was most frequently used since it was found early in the work that the dose was determined by the relation between the total quantity of spores and total quantity of toxicant present rather than by the concentration of either toxicant or spores alone. It was easily possible to obtain a dosage-response curve, with dosage based on the amount of toxicant taken up per unit of spore weight, by using only one concentration of fungicide but varying the quantity of spores.

With the methods employed it was not practical to use exposure periods

of less than about 15 seconds, although, as the data will show, much fungicide obviously was taken up in less than this time.

To determine the effect on germination, the fungicidal solution was removed at the end of the treatment period, the spores resuspended in water, an appropriate aliquot taken, and the spores subjected to conditions conducive to germination as described earlier. With all three fungicides, resuspension in water did not result in removal of an appreciable quantity of the fungicide previously taken up.

DETERMINATION OF RADIOACTIVITY

Radioactivity was determined by the use of conventional scalers and end window type Geiger tubes with thin windows. The radioactive materials used were available with sufficiently high specific activity so that measurements could be made on the sample directly without interference from self-absorption. The material to be counted was placed in stainless steel or nickel-plated planchets one inch in diameter. Quantities of toxicant counted per sample usually varied from less than 1 microgram to 10 micrograms. Since the fungus spores treated were small and took up relatively large amounts of the fungicides on a weight basis, the radioactivity of the treated spores could also be determined without an effect from self-absorption. When a question arose as to whether there might be self-absorption, tests were run to determine whether increasing the quantity of material increased the counts proportionately.

During the first part of the studies samples were usually counted for five minutes. Later an automatic sample changer was available and counting involved measuring the time required to reach a definite number of counts. Usually 1024 counts were used and always at least two measurements and often three or four were made on each sample.

QUANTITY OF TOXICANT TAKEN UP AND RELATION BETWEEN DOSE AND GERMINATION

2-HEPTADECYL-2-IMIDAZOLINE

Rapid Uptake by Spores of Neurospora sitophila

Interaction between 2-heptadecyl-2-imidazoline and fungus spores is characterized by an extremely rapid uptake of the toxicant by the spores from dilute solutions. This was evident in the first experiment in which spores of *Neurospora sitophila* were exposed to 10 ml. of an aqueous solution of the imidazoline containing 2.16 p.p.m. The data are given in Table I. The amount of toxicant removed by the spores was determined by the decrease in activity. At the start of the test 2.16 micrograms gave 494 counts per minute. It is seen that at the first sample period, after five minutes of contact, 85 per cent or more of the toxicant had been removed

from solution by the spores. Further exposure for 70 minutes at room temperature and 41 hours at 1° C. only resulted in the uptake of an additional 5 to 8 per cent.

It is evident that the dose received by the spores in this experiment was determined by the amount of toxicant present since both 25 and 46 mg. of spores removed the same quantity in five minutes representing a dose,

TABLE I

RATE OF REMOVAL OF 2-HEPTADECYL-2-IMIDAZOLINE BY SPORES OF *NEUROSPORA SITOPIHILA* FROM 10 ML. OF AN AQUEOUS SOLUTION CONTAINING 2.16 P.P.M.

Time intervals, minutes	Rate of removal by various quantities of spores, mg.					
	46		25		0	
	C.p.m.	Per cent removed	C.p.m.	Per cent removed	C.p.m.	Per cent removed
0	—	—	—	—	494	—
5	65	87	74	85	502	None
15	51	90	48	90	401	19
35	39	92	40	92	324	34
75	49	90	27	95	306	38
A further 41 hrs. at 1° C.	40	92	36	93	238	52
Dose received by spores in 5 min. in p.p.m.	409		734			

respectively, of 734 and 409 p.p.m. on a spore weight basis. The true dose therefore was many times greater than the concentration of the external solution and an accumulation of over 300-fold took place in five minutes.

It will be noted that there was some loss of toxicant in the control tube. The imidazoline is surface active and adsorption on the walls of glass vessels has occurred in all the experiments with this material. However, there was no detectable adsorption on glass during the first five minutes of the experiment and during this time 85 to 87 per cent of the imidazoline was removed in the tubes in which spores were present. Removal by the spores is so rapid and the tendency of the imidazoline to be adsorbed on glass is sufficiently slower so that interference from this source is not serious in such experiments.

It is obvious that in this test neither the lower limit as to the time required for the uptake of the toxicant by the spores nor the upper limit as to the degree of accumulation that can occur had been reached and additional experiments were undertaken to cover these points.

The results of an experiment in which an attempt was made to reduce the time of contact as much as possible under the procedures used are summarized in Table II. Counts per minute are omitted from all tables in this

section except the first one since not the counts but the values calculated from the counts are of primary interest. It is seen that here with only 10 mg. of spores, 69 per cent of the available imidazoline was taken out in the first 15 seconds. Further exposure increased uptake only about another 8 per cent. No loss in the control occurred during the first 15 seconds but an appreciable reduction took place in the second period recorded. The time of exposure, as indicated in the first column of Table II, could be adhered

TABLE II
UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE BY 10 MG. OF SPORES OF *NEUROSPORA*
SITOPHILA EXPOSED TO 2.16 P.P.M. IN 10 ML. OF AQUEOUS SOLUTION

Time, seconds*	Per cent removed	
	Spores	Control
15	69	> 1
30	72	24
45	77	24
60	77	33
75	71	35
90	80	38
105	77	39

* Opportunity for adsorption on glass continues after centrifugation and it is therefore not possible to compare directly adsorption in controls of Tables I and II. See text for fuller explanation.

to only for the tube containing the spores, in which the exposure period was considered ended when centrifugation began. The time required for the centrifugation and withdrawal of the samples for determination of radioactivity was considerable and during this period opportunity for adsorption on glass continued in the control. Adsorption on glass is not equally important in the tube with the spores because most of the material has already been taken out by the spores before adsorption on glass becomes appreciable. The data suggest that much uptake by the spores takes place before 15 seconds. The affinity of spores of *N. sitophila* for this fungicide is thus of a high order indeed.

It is clear that the relative quantities of spores and toxicant that were used in the experiments covered in Tables I and II were such that saturation of the capacity of spores to take up imidazoline has not nearly been reached. Data showing the extremely large dose required before the uptake of additional quantities becomes lessened, as well as the dose necessary to reduce germination, are given in Tables III and IV. In the experiments reported in Table III, 40-, 20-, and 10-mg. lots of spores were exposed to 10 ml. of solution containing 2.16 p.p.m. of toxicant for periods of one minute and an additional two minutes, after which the remaining supernatant was removed, 10 ml. of water added and sufficient imidazoline to

TABLE III

CONTINUED ABILITY OF SPORES OF *NEUROSPORA SITOPHILA* TO TAKE OUT LARGE QUANTITIES OF 2-HEPTADECYL-2-IMIDAZOLINE ON REPEATED EXPOSURE TO AQUEOUS SOLUTIONS CONTAINING 2.16 P.P.M.

Exposure period	Time, minutes	Imidazoline removed by various quantities of spores (mg.) in 10 ml.							
		40		20		10		0	
		Per cent	P.p.m.	Per cent	P.p.m.	Per cent	P.p.m.	Per cent	Micrograms
First	1 3	75 81	405	78 79	855	72 78	1680	12 28	5.7
Second	1 3	77 76	415	80 81	875	71 76	1600	15 16	3.4
Third	1 3	76 76	410	76 77	830	71 75	1560	4 5	0.9
Fourth	1 3	72 69	390	75 71	810	66 68	1460	8 9	2.0
Fifth	1 3	71 72	393	75 76	815	71 72	1540	—1 3	0.5
Sixth	1 3	72 69	390	72 —	780	65 67	1440	—2 0	0.4
Totals			2403		4965		9280		12.1
Germination, per cent		82		65		19			

give a solution containing 2.16 p.p.m. Uptake was again determined for one minute and for a further two-minute period. This procedure was carried out six times. The spores continued to take up about 70 to 80 per cent of the available toxicant during the first minute of exposure even though they had already received high doses previously. There may be an indication of a very moderate decrease in the rate of uptake of the 10-mg. lot after exposure to three successive solutions of toxicant resulting in a dose of about 5000 p.p.m. of spore weight. Such a decrease was at best very slight and it is apparent that the toxicant continued to be taken up at a very high rate even though subsequent germination capacity had become impaired. The data at the bottom of the table indicate 65 per cent germination after a dose of 4965 p.p.m. had been received, and 19 per cent germination upon uptake of 9280 p.p.m. Further data on the effect of dose on germination are given in a separate section following.

The data in Table IV show how high the possible dose (i.e., the dose if all toxicant present were taken up by the spores) has to be before an appreciable quantity of imidazoline remains unabsorbed after a short exposure. The higher doses were achieved in the experiment reported in the

lower part of the table by exposing 1, 3, and 9 mg. of spores each to 10 ml. of a solution containing 21.6 micrograms of the imidazoline and in the upper part of the table by using 10 mg. of spores in each instance but by supplying 21.6, 101.6, 181.6, and 341.6 micrograms of toxicant in 10 ml. respectively. The data show that only when the amount of imidazoline present is more than 10,000 p.p.m. on a spore weight basis, is the rate of

TABLE IV
RAPID UPTAKE OF LARGE AMOUNTS OF 2-HEPTADECYL-2-IMIDAZOLINE
BY SPORES OF *NEUROSPORA SITOPHILA*

Maximum possible dose, p.p.m.	Amount taken up in p.p.m. of spore weight after various time intervals in minutes				Germination, per cent
	0.5	2.0	5.0	15.0	
2,160	1,525	1,555	1,675	1,744	85
10,160	6,690	7,220	7,595	8,085	24
18,160	11,700	13,100	13,800	14,300	14
34,000	13,600	21,300	25,600	26,300	10
	0.5		5.0		
2,400	1,738		1,850		91
7,200	3,415		5,035		66
21,600	6,385		13,160		13

uptake decreased, so that less than 50 to 70 per cent is taken up in 0.5 to 2.0 minutes. Even when the possible dose was 34,000 p.p.m., 40 per cent was taken up in 30 seconds and a further 23 per cent in 1.5 more minutes. The values given in the table were obtained by determining the decrease in radioactivity of the solutions. These figures were also checked by determining the radioactivity of an aliquot of the treated spores. The results obtained by the two methods agreed within the experimental error of the methods employed.

Tests with Other Species of Fungi

In addition to the studies undertaken with spores of *Neurospora sitophila*, experiments have also been carried out with a considerable number of other species with some emphasis on those important as plant pathogens. Data for 10 species including *N. sitophila* are given in Table V in which the quantity taken up in one minute by 7 to 10 mg. of spores suspended in 10 ml. of a solution containing 2.16 p.p.m. of the imidazoline is listed. It is seen that spores of some other species, including *Venturia inaequalis*, the apple scab fungus, for the control of which the imidazoline is widely used, also take up large amounts rapidly. It is obvious, however, that there are rather large differences among the various species. The species are listed in order based on the percentage of available toxicant taken

up, although in many instances the differences are small and the order might be slightly different if further data were obtained. However, it will be noted that certain species are much less active than the first five listed.

Since 2-heptadecyl-2-imidazoline is known to be surface active, the surface area per mg. of spores is given in the last column of Table V. A glance at these values in relation to the rate of uptake of the imidazoline by the various species makes it clear that other factors besides surface area play a dominant role. Thus the species with the largest surface area per mg., *Cephalosporium acremonium*, is fifth in its affinity for the imidazoline

TABLE V
QUANTITIES OF 2-HEPTADECYL-2-IMIDAZOLINE TAKEN UP BY FUNGUS SPORES
IN ONE MINUTE FROM SOLUTIONS CONTAINING 2.16 P.P.M.

Species	Spore wt., mg.	Toxicant taken up		Surface area per mg. of spores, cm. ²
		Per cent	P.p.m. of spore weight	
<i>Neurospora sitophila</i>	10	70	1,512	5.9
<i>Venturia pyrina</i>	10	65	1,404	6.4
<i>Monilinia fruticola</i>	10	64	1,380	5.1
<i>Venturia inaequalis</i>	8	56	1,513	5.6
<i>Cephalosporium acremonium</i>	10	49	1,060	27.5
<i>Glomerella cingulata</i>	10	30	650	8.4
<i>Stemphylium sarcinaeforme</i>	7	29	886	2.0
<i>Alternaria oleracea</i>	10	28	600	5.6
<i>Rhizopus nigricans</i>	10	16	350	11.1
<i>Aspergillus niger</i>	10	13	280	14.7

and the species with the second, third, and fourth largest surface areas rank tenth, ninth, and sixth, respectively.

The differences in affinity for the imidazoline are much larger than the data in Table V indicate. Data have already been presented showing that *N. sitophila* may take up much more in one minute than the 1512 p.p.m. of spore weight noted under the conditions of the experiments summarized in the table. By using conditions under which a larger dose is available, difference in uptake by various species becomes greater. Such results are apparent in data presented in Table VI. In this experiment, 10 mg. each of *N. sitophila*, *Monilinia fruticola*, *Glomerella cingulata*, and *Aspergillus niger* were exposed to aqueous solutions of the imidazoline containing 32.2 micrograms. The uptake was determined after two minutes and then a further 10 mg. of spores was added and the uptake determined after another two minutes. This was continued until a total of 50 mg. of spores of each species had been used in the experiment. As might be expected from the data of Table V the largest amount was taken up by *N. sitophila* and the least by *A. niger*. Further additions of spores up to a total of 50 mg. did

not appreciably increase the uptake of the imidazoline by *A. niger*, but the addition of 10 mg. to make up a total of 20 mg. increased the uptake by *G. cingulata* to equal that taken up by 10 mg. of *N. sitophila* in the first two-minute period. The uptake of *Monilinia fruticola* was intermediate between that of *N. sitophila* and *G. cingulata*. On adding a further 32.2 micrograms of the imidazoline *N. sitophila* again took up the largest amount in two minutes while that taken up by *A. niger* was still less than that removed by 10 mg. of *N. sitophila* in the first two minutes of exposure

TABLE VI
QUANTITIES OF 2-HEPTADECYL-2-IMIDAZOLINE TAKEN OUT OF SOLUTION IN 2
MINUTES FOLLOWING SUCCESSIVE ADDITIONS OF 10-MG.
LOTS OF SPORES OF FOUR SPECIES

Cumulative weight of spores, mg.	Cumulative uptake of toxicant, micrograms*			
	<i>Neurospora sitophila</i>	<i>Monilinia fruticola</i>	<i>Glomerella cingulata</i>	<i>Aspergillus niger</i>
10	22.8	21.1	12.0	10.4
20	25.3	24.0	24.9	9.7
30	25.5	24.2	24.1	10.7
40	26.6	24.9	26.4	12.0
50	27.7	24.5	26.0	11.9
50	55.7	47.0	48.3	16.4
	58.2	49.2	49.3	20.7

* 32.2 Micrograms in 10 ml. at the start of the test. Another 32.2 micrograms was added after 50 mg. of spores were present and additional uptake for two two-minute periods is shown in the lower portion of the table.

The difference between the quantity of the imidazoline that can be taken up by *N. sitophila* and *A. niger* is therefore very great.

Interaction between Spores of Neurospora sitophila and Aspergillus niger

The case of *Aspergillus niger* received special attention in these experiments since it was found that the addition of spores of *A. niger* to spores of *N. sitophila* resulted in a decreased uptake of imidazoline by the latter. Further investigations showed that the supernatant from *A. niger* spores when added to spores of *N. sitophila* retarded uptake. A number of experiments showing the marked effect of spores and extracts of spores of *A. niger* on the uptake of the imidazoline are summarized in Table VII. In the upper part of the table are shown the results of experiments in which mixtures of spores of *A. niger* and *N. sitophila* were used. It is seen that spores of *N. sitophila* were much retarded in their ability to take up the imidazoline, when spores of *A. niger* were also present. The quantity of the imidazoline used in these tests was low enough so that 1 mg. of *N. sitophila* spores could have taken up a high percentage of the toxicant

TABLE VII

EFFECT OF SPORES AND THE SUPERNATANT FROM SPORE SUSPENSIONS OF ASPERGILLUS NIGER ON THE UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE BY NEUROSPORA SITOPHILA

Type of test	Condition	P.p.m. of spore weight of toxicant taken up after various time intervals in minutes		
		0.5	1.5	6.5
Spore mixtures (10 mg.), per cent of <i>A. niger</i> with <i>N. sitophila</i>	100	487	602	736
	90	421	442	489
	50	569	628	618
	30	599	620	683
	10	1,280	1,253	1,343
	0	1,372	1,449	1,546
Supernatant from <i>A. niger</i>	<i>N. sitophila</i> spores suspended in supernatant for one hr. before test	881	1,109	1,239
	Suspended during test	313	464	503
	Water control	1,319	1,428	1,528
Various quantities of <i>A. niger</i> supernatant added, ml. to 10 ml., <i>N. sitophila</i> spores	9	363	454	528
	3	881	881	925
	1	1,028	1,002	1,248
	0	2,497	2,629	2,678
	1	1,020	1,222	1,450
	0.3	1,561	1,770	1,963
	0.1	1,693	2,117	2,365
	0	2,257	2,459	2,558
Effect of washing of <i>A. niger</i> spores on uptake by <i>A. niger</i> , No. of washings	4	621	727	869
	2	652	837	798
	1	514	710	852
	0	697	663	810

available in a minute or less but this did not happen even for 7 mg. of spores, when spores of *A. niger* were permitted to interfere. The data in the next three sections show that the supernatant from washings of *A. niger* spores also markedly retards uptake and that quite small amounts of this supernatant were effective. Even when spores of *N. sitophila* were merely suspended in this supernatant for one hour and the supernatant then discarded, the retarding effect was still operative. Although the factor or factors producing this effect are prominent in washings from *A. niger* spores, they persist in the spores themselves after repeated washings as shown in the last part of Table VII. Four washings of *A. niger* spores did not increase the uptake of the imidazoline by spores of this species over that of spores not treated. As will be pointed out in a later section, the supernatant from *A. niger* also retards the uptake of silver but, on the other hand, cerium is taken up much more rapidly by *A. niger* than by *N. sitophila*.

Quantity of 2-Heptadecyl-2-imidazoline Taken Up Under Conditions of Slide-Germination Test

In the slide-germination test, in which 50,000 to 100,000 spores per ml. are exposed to solutions of toxicant for 24-hour periods, the quantity of toxicant available on a spore weight basis is even larger than in most of the experiments reported above. In order to see how much of the imidazoline was taken up under these conditions, tests were carried out with 100,000 spores per ml. of *Glomerella cingulata*, *Neurospora sitophila*, and *Rhizopus nigricans* in a solution of the imidazoline containing 2.16 p.p.m. The data are given in Table VIII. Quantities of the imidazoline equivalent

TABLE VIII

QUANTITIES OF 2-HEPTADECYL-2-IMIDAZOLINE TAKEN UP BY SPORES WHEN EXPOSED TO 2.16 P.P.M. FOR 24 HOURS AT A SPORE CONCENTRATION OF 100,000 PER ML.

Species	Wt. of spores in 10 ml., micrograms	Toxicant, % of spore wt.	Toxicant taken up, p.p.m. of spore wt.
<i>Glomerella cingulata</i>	283	7.6	7,400
<i>Neurospora sitophila</i>	347	6.2	20,700
<i>Rhizopus nigricans</i>	57	37.9	52,600

to from 7400 to 53,000 p.p.m. of spore weight were taken up. Since only 2.16 p.p.m. was present in the external solution, accumulation of over 20,000-fold took place with spores of *Rhizopus nigricans*. Data such as these emphasize the high doses on a spore weight basis presented to spores in the slide-germination test. Also, it is apparent that when it is desired to compare various chemicals as to their innate toxicity, information obtained in this way can be misleading if some of the chemicals under test are accumulated and others are not.

Relation between Dose and Germination

Some germination data in relation to amount of the imidazoline taken up by spores of *Neurospora sitophila* are given in Tables III and IV. It will be noted that the doses reached were obtained by three different methods: (a) by repeated treatments of spores with a given concentration of toxicant, (b) by varying the relative spore weight in the tests, and (c) by keeping the spore weight constant but increasing the concentration of toxicant applied. In spite of this, all points fall essentially on the same line when plotted on logarithmic-probability paper (Fig. 1 A). The resultant value for the concentration of toxicant required per unit of spore weight to prevent germination of 50 per cent of the spores, i.e., the ED₅₀, was 5100 p.p.m.

In an earlier test in which a dosage-response curve was obtained for the imidazoline and spores of *Neurospora sitophila* by using the same concen-

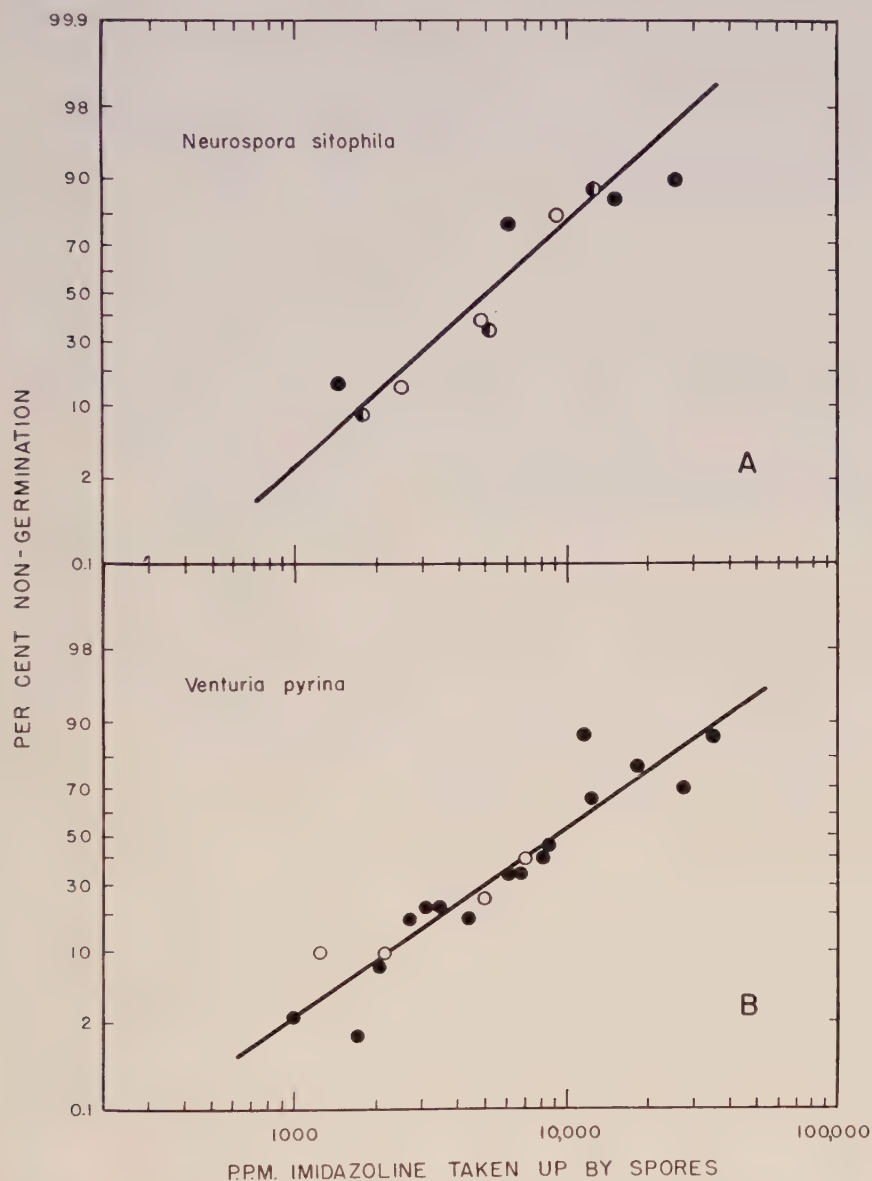


FIGURE 1. Dosage-response curves for the effect of 2-heptadecyl-2-imidazoline, on a spore weight basis, on (A) *Neurospora sitophila*, and (B) *Venturia pyrina*. Different kinds of circles indicate different experiments.

tration of toxicant throughout, but varying the quantity of spores, an ED₅₀ value of 6500 was obtained. In this test doses as high as 35,000 p.p.m. of spore weight were required to prevent 95 per cent of the spores from germinating. A dosage-response curve was also obtained for spores of *Venturia pyrina*. This is shown in Figure 1 B. The ED₅₀ value for this species was found to be 9300 p.p.m.

FERRIC DIMETHYLDITHIOCARBAMATE

Experiments with ferric dimethyldithiocarbamate were not so extensive as were those with other materials used in these studies. However, sufficient information was obtained to show that this fungicide also is taken up rapidly by fungus spores. The data are given in Table IX. The

TABLE IX
UPTAKE OF FERRIC DIMETHYLDITHIOCARBAMATE BY 50-MG. LOTS OF
FUNGUS SPORES WHEN EXPOSED IN AQUEOUS SOLUTIONS

Species	Concn. of toxicant, p.p.m.	Time, minutes	Toxicant taken up	
			Per cent of that available	P.p.m. of spore wt.
<i>Neurospora sitophila</i>	28	1	48	1,363
		4	65	1,574
		10	66	1,583
		40	68	1,585
<i>Saccharomyces cerevisiae</i>	13		70	920
<i>Cephalosporium acremonium</i>	11	10	45	500
<i>Stemphylium sarcinaeforme</i>	13		35	460

fungicide was in aqueous solution in all the tests. Fifty mg. of spores or of dried yeast cells (*Saccharomyces cerevisiae* Hansen) were exposed to 5 ml. of solution of toxicant at the concentrations shown in the table. Manyfold accumulation occurred and it is clear that with this material also, dosage on a spore weight basis is much higher than the concentration of the external solution would indicate.

SILVER

As a representative of a metal with fungicidal properties silver was chosen for inclusion in these studies. It is the most toxic of all metals when tested by the slide-germination technique (4, 11) and a suitable radioisotope, Ag¹¹⁰, is available. Studies reported by Marsh (5) some years ago showed that copper is concentrated in spores of *Monilinia fructicola* as

much as 4000-fold and that high concentrations on a spore weight basis are necessary for germination to be affected.

Rate of Uptake by Fungus Spores

Data for the rate of uptake of silver for spores of four species are given in Table X. Although the concentration of the external solution was only 1.04 p.p.m. of silver, the spores took up as much as 2000 p.p.m. in 30 sec-

TABLE X
RATE OF UPTAKE OF SILVER BY FUNGUS SPORES WHEN EXPOSED TO 1.04 P.P.M.

Species	Spore wt., mg.	Ag as p.p.m. of spore weight				Germination, %	
		Maximum dose possible	Amount taken up after various intervals in minutes				
			0.5	120			
<i>Monilinia fructicola</i>	2.5	4,160	2,124	2,580		0	
	5	2,080	1,532	1,312		0	
	10	1,040	937	833		6	
	20	520	444	385		12	
	40	260	243	231		67	
	80	130	120	128		85	
<i>Alternaria oleracea</i>	10	1,040	744	954		11	
	20	520	446	490		31	
	40	260	231	246		76	
	80	130	121	124		91	
				0.5	1.5	6.5	26.5
<i>Aspergillus niger</i>	10	1,040	482	534	675	817	26
	20	520	312	331	384	431	67
	40	260	169	195	207	226	100
	80	130	100	110	112	120	96
<i>Neurospora sitophila</i>	5	2,080	1,084	1,476	1,714	1,754	—
	10	1,040	583	738	699	714	—
	20	520	408	382	342	335	—

onds. With all species, covering possible doses of from 130 to 4160 p.p.m. of spore weight, 50 to 90 per cent of the available silver was removed from solution in the first 30 seconds. Further periods of contact resulted in additional uptake but in most instances the quantities were minor compared to the uptake in the first 30 seconds. There was no loss of silver from solution in the control tubes.

Effect on Germination

The relation between the quantity of silver taken up and the effect on germination capacity for spores of *Monilinia fructicola*, *Alternaria oleracea*,

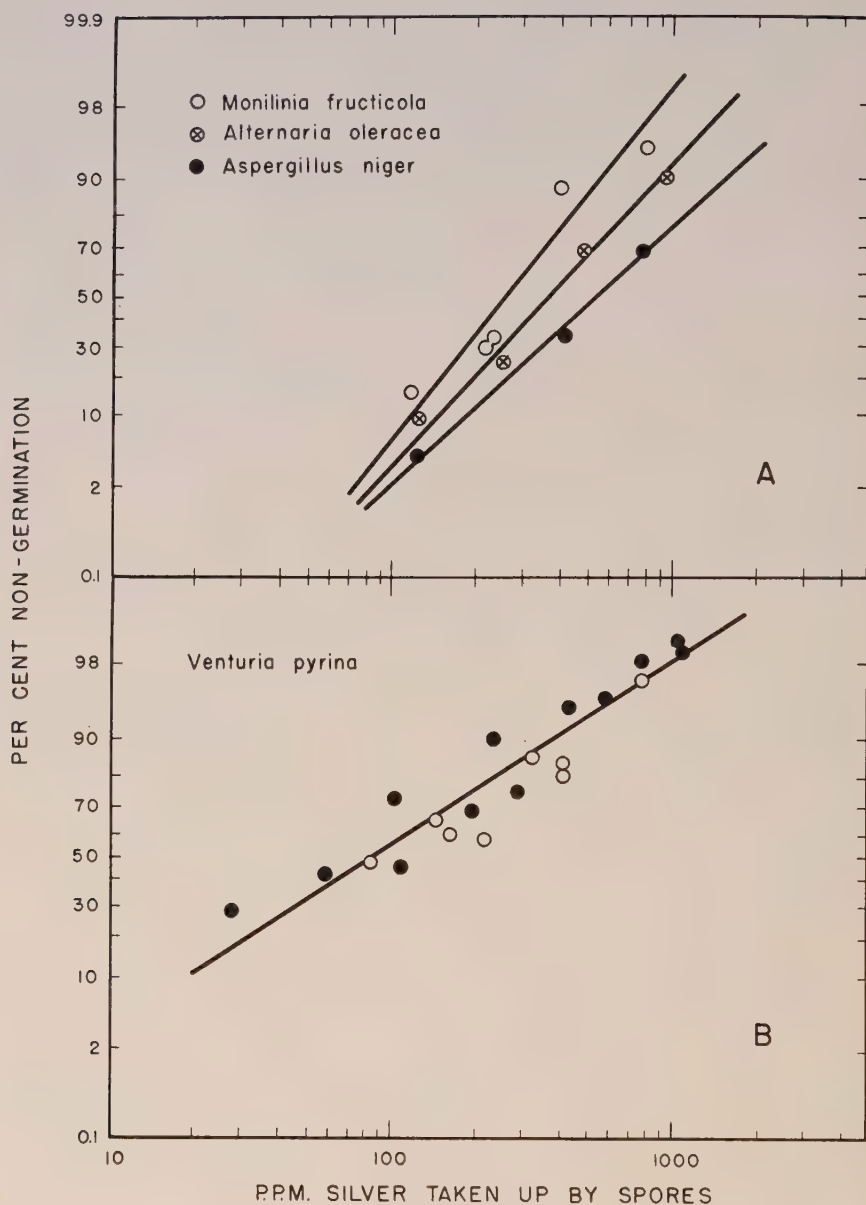


FIGURE 2. Dosage-response curves for the effect of silver on a spore weight basis on (A) *Monilinia fruticola*, *Alternaria oleracea*, and *Aspergillus niger*, and (B) *Venturia pyrina*. Different kinds of circles indicate different experiments.

Aspergillus niger, and *Venturia pyrina* is shown in Figure 2 in the form of dosage-response curves. The ED₅₀ values on a spore weight basis varied from 85 p.p.m. for *Venturia pyrina*, the most sensitive species, to 250, 360, and 540 p.p.m. for the other three species in the order named above. Since the concentration of the external solution was only 1.04 p.p.m., accumulation varying from 75- to 520-fold was necessary before 50 per cent inhibition of germination occurred. To prevent 95 per cent of the spores of *A. niger* from germinating, 2000-fold accumulation was necessary.

Effect of Supernatant from Spores of Aspergillus niger on Uptake by Neurospora sitophila

Supernatant from spores of *Aspergillus niger* which affected the uptake of 2-heptadecyl-2-imidazoline by spores of *Neurospora sitophila* was also found to have a similar effect on the uptake of silver. Data illustrative of the results obtained in such tests are given in Table XI. It is seen that the

TABLE XI
EFFECT OF SUPERNATANT FROM ASPERGILLUS NIGER SPORES ON
UPTAKE OF SILVER BY SPORES OF NEUROSPORA SITOPHILA

Quantity of supernatant from <i>A. niger</i> in 10 ml., ml.	Ag as p.p.m. of spore weight			
	Maximum dose possible	Uptake after various time intervals in minutes		
		0.5	1.5	6.5
8	1,030	0	45	83
3		175	259	274
1		320	448	622
0		261	640	841

effect is quite marked and even a small quantity of supernatant persisted in its influence during the course of the experiment. The actual dry weight of material in the *Aspergillus niger* supernatant used was very small. Whether the effect on the uptake of imidazoline and of silver is the result of a simple chemical reaction between some constituent of the supernatant from *A. niger* and the toxicants in question remains to be determined. As will be shown in the section below, reporting experiments with cerium, spores of *A. niger* take up this ion much more readily than spores of *N. sitophila*.

CERIUM

Cerium was chosen as still another toxicant to be tested since it is one of a number of rare earth elements which have shown toxicity to fungus spores in slide-germination tests (4). Since cerium has a relatively low order of toxicity the tests were carried out with solutions containing 10 p.p.m. of cerium as cerous sulfate. The activity was furnished by carrier free

TABLE XII

RATE OF UPTAKE OF CERIUM BY SPORES OF A NUMBER OF SPECIES OF FUNGI WHEN EXPOSED TO 10 P.P.M.

Species	Spore wt., mg.	Cerium as p.p.m. of spore wt.					Germination, %
		Max. dose possible	Amount taken up after various time intervals in minutes				
			0.5	1.5	6.5	36-66	
<i>Neurospora sitophila</i>	10	10,000	827	887	808	970	100
	30	3,333	640	839	670	733	100
<i>Alternaria oleracea</i>	11.6	8,620	2,255	3,820	5,990	7,110	100
<i>Neurospora sitophila</i>	10		630	590	910	480	100
<i>Aspergillus niger</i>	10		4,500	5,220	5,400	5,670	100
<i>Sclerotinia fructicola</i>	10	10,000	1,400	1,450	700	840	—
<i>Neurospora sitophila</i> and <i>Aspergillus niger</i>	5 each		3,530	3,170	3,130	3,580	—
<i>Neurospora sitophila</i>	10			753*	999*	947*	
<i>Aspergillus niger</i>	10			4,800	4,827	4,735	
<i>Sclerotinia fructicola</i>	10	10,000		1,475	1,844	1,804	
<i>Glomerella cingulata</i>	10			508	1,201	572	
<i>Neurospora sitophila</i>	10	10,000	647**	356**	960**	982**	99
<i>Aspergillus niger</i>	10	10,000	7,230	9,340	9,395	9,500	100
	20	5,000	4,785	4,915	4,755	4,872	98
<i>Aspergillus niger</i>	10	10,000	7,625†	8,260†	8,270†	8,440†	98
	30	3,333	3,235	3,260	3,300	3,280	74
	90	1,111	1,080	1,080	1,100	1,100	77
<i>Neurospora sitophila</i>	30	3,333	260	425	325	402	100

* Values in these columns represent time periods of 2, 15, and 915 minutes respectively.

** Time periods of 1, 6, 16, and 106 minutes respectively.

† Time periods of 0.5, 1.0, 6.5, and 16.5 minutes for these columns.

$Ce^{144}Cl_3$. The data from a number of experiments on the uptake of cerium are given in Table XII. Appreciable uptake in 30 seconds, as was found for silver and the imidazoline, also occurred with cerium. In many of the experiments no further cerium was removed from solution after the first 30 seconds; in some instances there was an apparent release of cerium back into the solution. In these tests in which the amount of toxicant available was considerably higher than with silver and the imidazoline, the percentage of toxicant taken out during the first 30 seconds was less, although values as high as 75 per cent were reached with *A. niger*.

The results with cerium offer some interesting contrasts among the spores of different species as compared to their reaction to other toxicants. In general, uptake was much less than with the imidazoline although larger quantities were available. Spores of *Alternaria oleracea* and *Aspergillus*

niger were exceptions in that they took up relatively large amounts. Surprisingly, spores of *Neurospora sitophila* which take up large amounts of sulfur (10), 2-heptadecyl-2-imidazoline, and silver, took up comparatively little cerium, and spores of *Aspergillus niger* which take up sulfur very slowly, and even affect the uptake of the imidazoline by other species, took up cerium actively. Although concentrations of about 7500 p.p.m. were reached with spores of this species in 30 seconds, cerium was not toxic. Neither was it toxic to spores of *Alternaria oleracea* in a single test

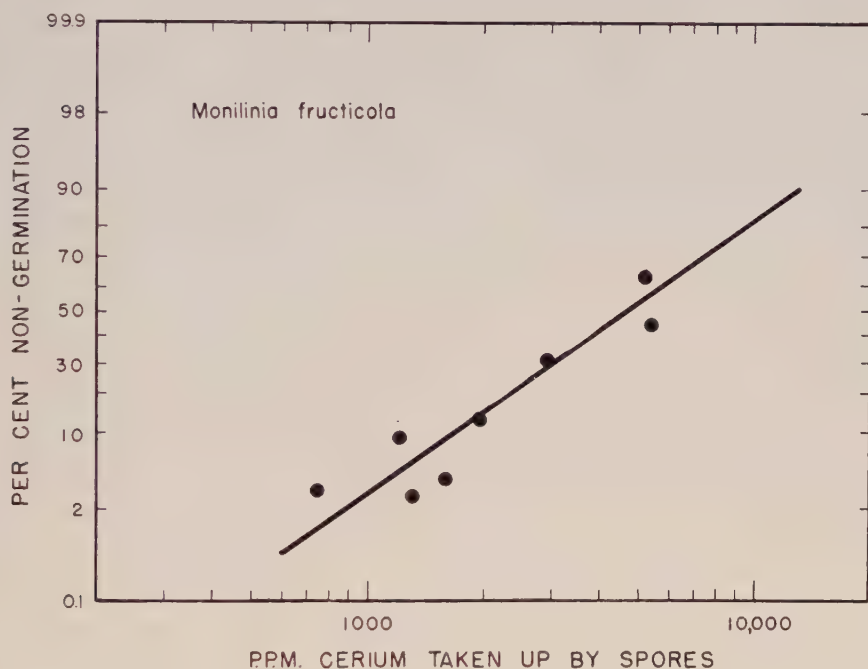


FIGURE 3. Dosage-response curves for the effect of cerium on a spore weight basis on *Monilinia fructicola*.

in which 7000 p.p.m. was reached in the spores. Although spores of *Monilinia fructicola* did not take up as much as those of some other species, cerium was toxic to these spores. A dosage-response curve obtained by treating lots of 1, 5, 25, and 125 mg. of spores with 10 ml. of a solution containing 10 p.p.m. for two hours, and again for an additional one and one-half hours, is shown in Figure 3. The ED₅₀ value was found to be about 4600 p.p.m.

SLOPE OF DOSAGE-RESPONSE CURVES

It will be noted in Figures 1 to 3 that the data obtained can be plotted as a straight line on logarithmic-probability paper. The slopes, i.e., $1/\log$.

(ED₈₄/ED₅₀), of these curves range from approximately 2.0 to 4.8. Data obtained recently and in earlier studies (3) for spores of some of the same species and chemicals by the slide-germination test, in which the external concentration of the toxicant was plotted as the dose, gave slopes which were more or less similar. This range of slopes also agrees with that found earlier for a majority of a number of different kinds of chemicals tested on the spores of the same species by the slide-germination method.

DISCUSSION

The most important conclusion to be drawn from the results of the tests reported in this paper and the preceding one concerned with sulfur (10), seems to be that the actual dose received by the spores is much higher than the concentration of the applied solution would indicate. The degree of accumulation, that is, the increased concentration associated with the spores as compared with the external solution, varied with the species and with the toxicant. With 2-heptadecyl-2-imidazoline, it could be as high as 10,000-fold or more, with silver over 2000-fold, in limited tests with ferric dimethyldithiocarbamate 70-fold, and with cerium 800-fold. With sulfur, if one considers the total quantities of hydrogen sulfide which are given off as the dose of sulfur received, the effective doses on a spore weight basis could be as high as 60,000 p.p.m. without resulting in complete loss of germination capacity (10). Insofar as the fungitoxicans included in these tests indicate, the ED₅₀ values determined in the slide-germination tests (1, 2), which are based on the concentration of external solution, may therefore be far from the values for innate toxicity expressed on a spore weight basis.

Actually, as is seen from the data in Table VIII, the amount of toxicant available in this test is usually extremely high and may be equal to or greater than the weight of spores present. When chemicals are compared as to their efficacy by this technique the results obtained, therefore, confound innate toxicity and ability to accumulate the toxicant by the spores. Insofar as one may be interested in the results of such a test without regard to this confounding, the conclusions will be satisfactory. On the other hand, if there is an interest in innate toxicity, that is, toxicity based on the dose received by the spores, the results may be misleading. It is obvious that in such a test, the materials not accumulated are at a disadvantage even though their innate toxicity may be quite high. It is possible, therefore, that in screening procedures compounds with potential value as active toxicants may be discarded in favor of less toxic materials which are accumulated to a high degree. This is readily apparent when one recalls that the imidazoline used in these studies may accumulate 10,000-fold or more. A competitive chemical which does not accumulate at all would therefore have to be 10,000 times as toxic to be considered equally attractive.

Determination of the actual dose on a weight basis has been difficult in the past because of the small quantities involved and was possible in the studies reported here because of the availability of radioactive isotopes. However, whether or not extensive accumulation takes place can be determined by techniques not employing radioisotopes. Thus, if accumulation does not occur, results on the effect on germination capacity should be independent of the relative quantities of toxicant and spores present in the test solution. If accumulation takes place it is possible to detect the occurrence of same by testing the toxicity of solutions which have previously been in contact with fungus spores. By the use of these techniques other fungicides not available in labeled form have been tested and preliminary results indicate that accumulation occurs with other fungitoxics in addition to those covered in the present publication. One of course hopes that as such studies are continued, fungitoxic chemicals which do not accumulate will be disclosed. It is suggested that in a screening program in which it is desired to discern relationships between the structure of chemicals and their fungitoxic effectiveness due emphasis should be placed on the fact that in the slide-germination tests innate toxicity and accumulation are confounded.

The results reported show clearly that there are large differences in the capacity of spores to take up various toxicants. Furthermore there are also wide variations in the quantity of toxicant necessary to inhibit germination. The specificity of fungicides, which is becoming increasingly evident as more fungicidal materials are being discovered, is therefore the result of a combination of differences in the quantity of toxicant taken up by spores of various species and differences in the effectiveness of the toxicant after being taken up. There may also be effects of spores of one species on those of another when exposed to a toxicant at the same time as exemplified in the results obtained with spores of *A. niger*.

With the exception of sulfur (10) which is the least soluble of all the fungicides included, large quantities of the toxicants, in fact often the major quantities involved, are taken up during the first 30 seconds of exposure. The question arises as to whether the materials are really taken in by the spores or merely adsorbed on the surface. Of the materials included in the tests, 2-heptadecyl-2-imidazoline is especially surface active and is adsorbed to some degree on glass. However, large quantities of the non-surface active toxicants, such as silver and cerium, are also removed from solution rapidly. The quantities involved are such that if adsorption or reaction with materials on the surface were the only factors operating, layers several molecules thick would result. One would therefore expect that previous exposure to one toxicant resulting in covering of the spore surface with a layer a number of molecules thick would interfere with reaction with other toxicants subsequently applied. Preliminary results in

experiments in which treatment by one toxicant was followed by treatment with another indicate a marked lack of influence of one upon the other. There would thus appear to be some doubt as to whether surface phenomena play as large a role as one might at first suspect.

SUMMARY

The rate of uptake, the dose received, and the toxicity on a spore weight basis were determined for 2-heptadecyl-2-imidazoline, ferric dimethyldithiocarbamate, silver, and cerium with the use of radioactive tracer techniques. The fungi used included *Monilinia fructicola*, *Alternaria oleracea*, *Stemphylium sarcinaeforme*, *Glomerella cingulata*, *Aspergillus niger*, *Rhizopus nigricans*, *Cephalosporium acremonium*, *Neurospora sitophila*, *Venturia inaequalis*, and *V. pyrina*. Not all fungi were tested with each chemical but extensive comparisons have been made as to the differences in affinity for and toxicity of the fungicides for spores of various species.

The dose received on a spore weight basis was many times that indicated by the concentration of the external solution. With the imidazoline the degree of accumulation amounted to 10,000-fold or more. Over a wide range the concentration of toxicant taken up by the spores was determined by the relative quantities of chemical and spore weight rather than by the concentration of either.

With all the toxicants examined uptake was surprisingly rapid. In most instances a large percentage of the total quantity that was to be taken up by the spores was taken up in the first 30 seconds or less. The rate at which spores of *Neurospora sitophila* took up the imidazoline was not lowered appreciably until the relative quantities of spores and toxicant were such that doses considerably above 10,000 parts per million of spore weight were involved. Spores of some species such as those of *Aspergillus niger* were considerably less active in taking up the imidazoline; actually spores of this species and the supernatant from suspensions of these spores retarded the uptake by spores of *Neurospora sitophila*.

The innate toxicity of the imidazoline to spores was relatively low but, because of the rapid rate of uptake and marked accumulation from dilute solutions, control of germination capacity is readily attained. The ED₅₀ values on a spore weight basis were 9250 and 5800 p.p.m. for *Venturia pyrina* and *Neurospora sitophila*, respectively.

Silver was the most toxic on a spore weight basis of all the materials tested. ED₅₀ values from 85 to 540 p.p.m. were obtained with spores of four species of fungi. These doses were taken up from solutions containing 1.04 p.p.m.

Spores of different species differed markedly in their reaction to cerium. Most rapid and extensive uptake occurred with spores of *Alternaria oleracea* and *Aspergillus niger* but germination remained essentially unaffected.

Less toxicant was taken up by spores of *Monilinia fruticola* but germination was retarded. The ED₅₀ value for the latter was found to be 4600 p.p.m.

These results emphasize the importance of determining dosage, whenever possible, on a spore weight basis. When different chemicals are compared as to their effectiveness on the basis of the concentration of the applied solutions, no distinction is possible between differences in degree of accumulation and innate toxicity. For many purposes differentiation between the two effects is very desirable.

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THE ROLE OF AUXIN IN PLANT FLOWERING. I. A QUANTITATIVE METHOD BASED ON PAPER CHROMATOGRAPHY FOR THE DETERMINATION OF INDOLE COMPOUNDS AND OF 3-INDOLEACETIC ACID IN PLANT TISSUES¹

A. J. VLITOS AND WERNER MEUDT

High levels of auxin in the plant have been postulated to be unfavorable for the flowering of plants (6, 9). This view has been based upon the many examples of suppression of flowering in short-day plants by applications of synthetic plant-growth regulators (5, 7, 8, 11, 12, 14, 15, 16), and, conversely, upon the fact that some antiauxins can stimulate the flowering of certain plant species under photoperiods not conducive to floral initiation (4, 10, 17).

The present paper is concerned with preliminary experiments designed to investigate the role of 3-indoleacetic acid in the flowering mechanism. Development of a specific, quantitative chemical method for indole compounds in general is extremely desirable if a critical examination of auxin levels in relation to flowering is to be made.

Paper chromatography as an analytical tool in auxin physiology has been demonstrated to be suitable by Jerchel and Müller (13) and by Bennet-Clark *et al.* (1). The present work is an attempt to extend the number of indole compounds subjected to paper chromatography by either of these investigators and, in addition, to increase the quantitative precision of the technique by employing densitometer readings of spots developed with *p*-dimethylaminobenzaldehyde. The technique has been found to be rapid, sensitive, and reproducible; hence it should serve as a valuable analytical tool in elucidating auxin metabolism in plant cells.

DESCRIPTION OF METHOD

The following procedures were employed to develop a standard calibration curve. 3-Indoleacetic acid (IAA) was dissolved in isopropyl alcohol. Drops (2.5 microliters) containing 2.5, 5, 10, 20, 40, and 80 micrograms IAA were applied by means of a capillary pipette to a line drawn 2.5 cm. from the edge of a strip of Whatman No. 1 filter paper (50×46.5 cm.). The drops were applied at least 3 cm. apart from each other and were air-dried for five minutes before the edge of the paper strip was placed in a trough containing isopropyl alcohol-ammonia-water (80:5:15 v/v). After 15 hours the solvent traveled upwards and over the bar in an ascending-

¹ The authors are indebted to Dr. Gunter Zweig for his valuable assistance in this work.

descending type chamber as described by Block, Le Strange, and Zweig (3). The paper was removed from the solvent, dried in a hood, sprayed with a 1 per cent solution of *p*-dimethylaminobenzaldehyde in *N* HCl, and allowed to dry. The above operations were done at room temperatures (22° to 25° C.).

The optical density of the deep, blue spots which develop can be determined by means of a Densichron transmission densitometer (Welch Mfg. Company). If the logarithm of the concentration of IAA is plotted against

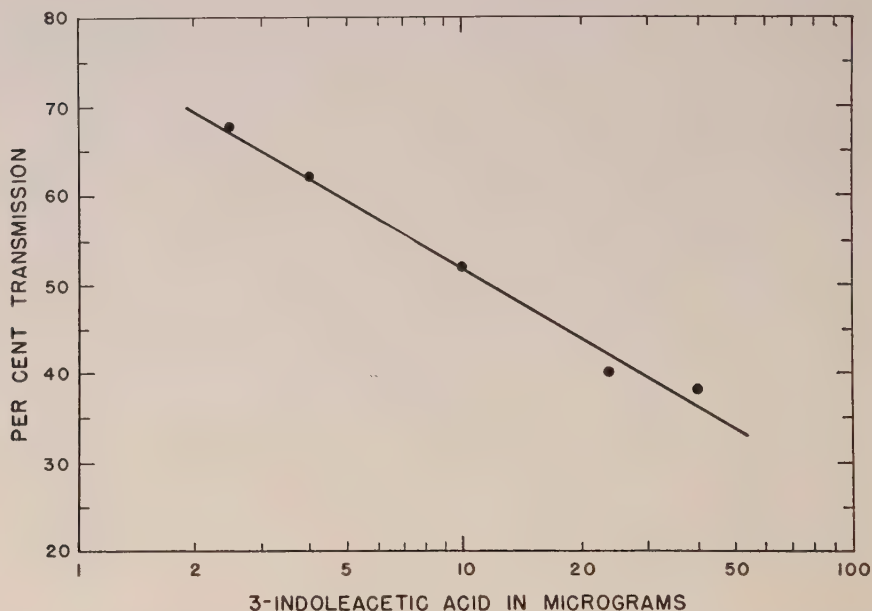


FIGURE 1. Typical calibration curve for 3-indoleacetic acid based on densitometer reading of spots developed with *p*-dimethylaminobenzaldehyde on paper chromatograph.

the per cent transmission of light a straight-line relationship is attained (Fig. 1). As the logarithm of concentration is increased there is a corresponding decrease in per cent transmission.

If a densitometer is not available, it is possible to employ the area-spot method (3) which has been found to be applicable for the quantitative estimation of IAA (1). However, the densitometer was found to give much more accurate results than the area-spot method.

SPECIFICITY OF THE METHOD

The reliability of any quantitative method for IAA ultimately rests upon its specificity. Therefore, the following indole compounds have been chromatographed singly and in combination by the above technique; IAA,

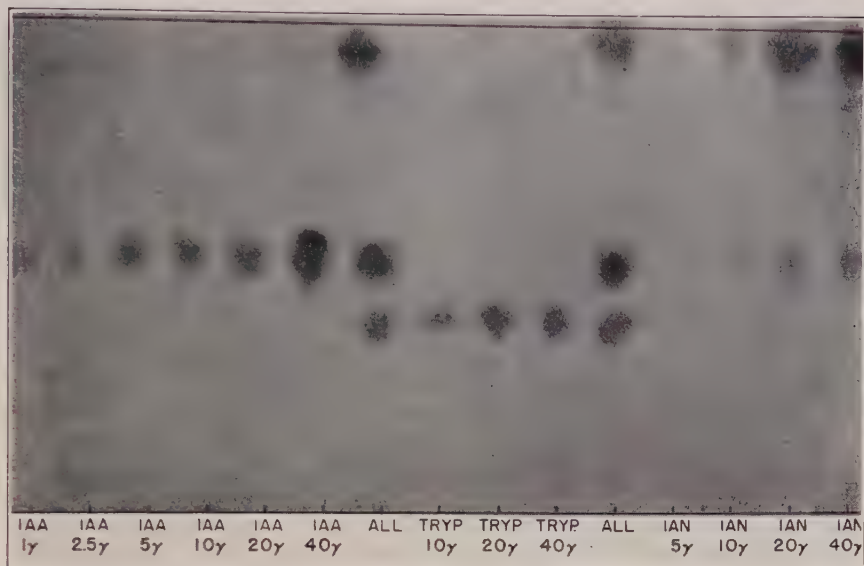


FIGURE 2. Paper chromatograph of 3-indoleacetic, tryptophan, and 3-indoleacetonitrile. From left to right: IAA 1 to 40γ, mixture of all three compounds (ALL), tryptophan 10 to 40γ, and 3-indoleacetonitrile (IAN) 5 to 40γ. Contamination of 3-indoleacetonitrile with IAA can be detected on the paper. (In solvent for 15 hrs.)

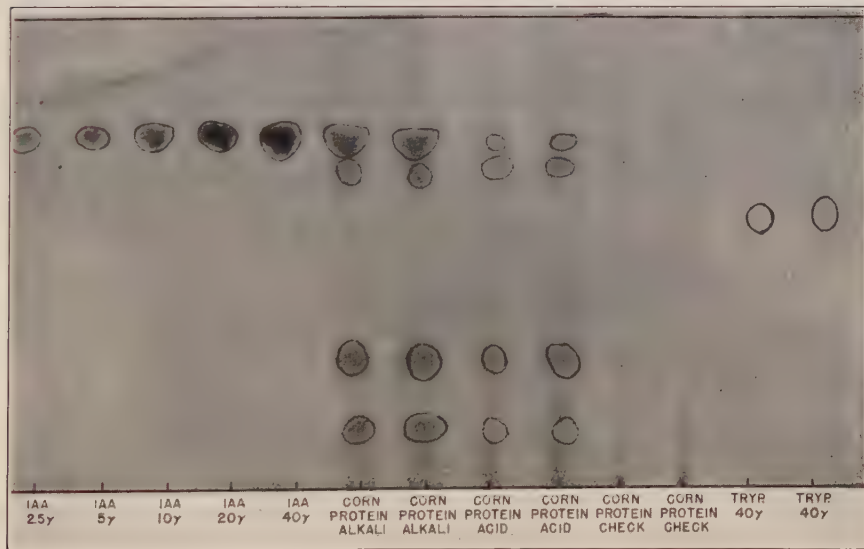


FIGURE 3. Paper chromatograph showing from left to right: IAA (2.5 to 40.0γ), alkaline and acid hydrolysates of corn protein, non-hydrolyzed corn protein (check), and tryptophan (40γ). Note less IAA from acid hydrolysis. (In solvent for 12 hrs.)

indole, 3-indolealdehyde, 3-indolebutyronitrile, 3-indoleacetonitrile, 3-indolecarboxylic acid, 3-indoleacrylic acid, 3-indolelactic acid, 3-indoleethylamine hydrochloride, 3-indolepropionic acid, and tryptophan. Photographs of chromatographs (Figs. 2, 3, and 4) illustrate the separation

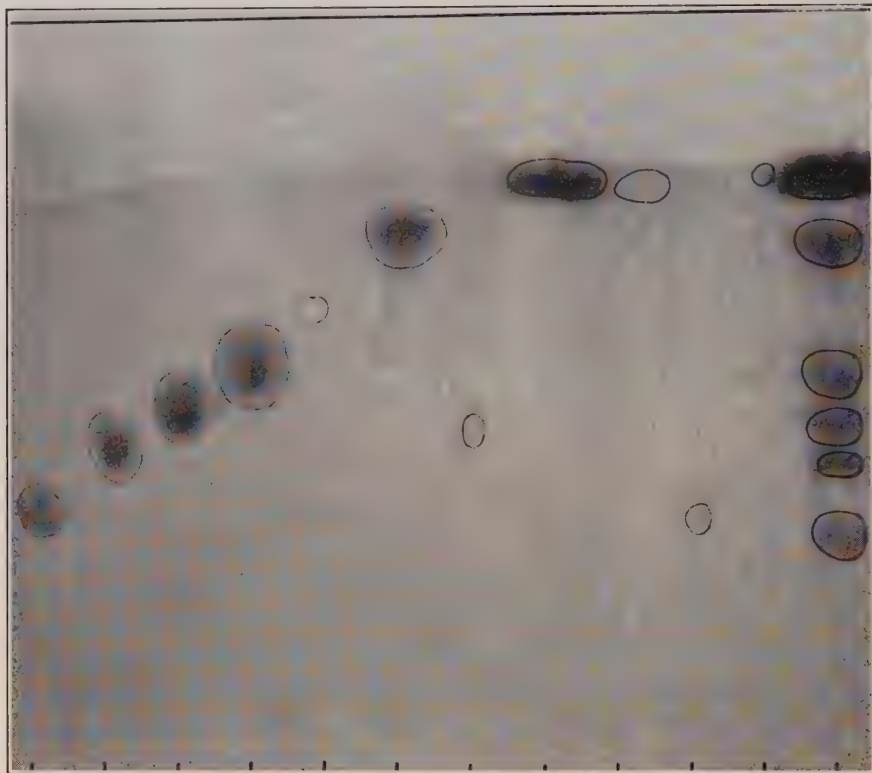


FIGURE 4. Paper chromatograph showing from left to right: tryptophan (20γ) Rf .34, IAA (20γ) Rf .43, 3-indolepropionic acid (20γ) Rf .49, 3-indolebutyric acid (20γ) Rf .53, 3-indolecarboxylic acid (40γ) Rf .62, 3-indoleethylamine HCl (20γ) Rf .72, 3-indolealdehyde (40γ) Rf .46, 3-indolebutyronitrile (40γ) Rf .79, indole (40γ) Rf .78, 3-indolelactic acid (40γ) Rf .33, 3-indoleacrylic acid (20γ) Rf .80, and separation of a mixture of these compounds from a plant extract. (In solvent for 24 hrs.)

of these compounds on paper. The indole nitriles are distinguished from indole by the blue color in nitrile spots as contrasted to red for indole. Rf values appear in Figure 4.

APPLICATION OF THE METHOD TO ANALYSES OF PLANT TISSUES

Preliminary experiments indicate that the method is free of interference from other soluble compounds which may be carried along with ether extractions of IAA from plant tissues. Berger and Avery's (2) zein-like, auxin-yielding protein fraction was isolated from seeds of sweet corn

(*Zea mays* L. var. Golden Cross Bantam) and IAA was determined to be present following alkaline hydrolysis. Tissues of tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) have been extracted with peroxide-free ether following treatment of the plants with known amounts of IAA, and the extracts have yielded measurable quantities of IAA after alkaline hydrolysis of the tissues. Negative tests for IAA have been obtained in ether extracts of kilogram quantities of leaf and stem tissues of soybean (*Glycine max* ^{Desf.} ~~Meer.~~ var. Biloxi), barley (*Hordeum vulgare* L. var. Wintex), tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth), spinach (*Spinacia oleracea* L. var. Nobel), and tomato. However, upon alkaline hydrolysis of barley and tobacco tissues IAA can then be determined to be present in the extracts. These results lend support to the contention that IAA, in the tissues examined by this technique, is present in a "bound" form. "Free" IAA is not present in amounts detectable by this method. These results will be presented and discussed more fully in a forthcoming publication.

The successful application of paper chromatography for analyses of auxin content in plant tissues depends upon satisfactory extraction methods. As yet no single method for extracting auxin from plant tissues has been found to be satisfactory for all species of plants. The present method can therefore be applied to best advantage only if the substances to be determined are extracted satisfactorily.

SUMMARY

A quantitative chemical technique is described based on paper chromatography for the determination of IAA, indole, 3-indolealdehyde, 3-indolebutyronitrile, 3-indoleacetonitrile, 3-indolecarboxylic acid, 3-indoleacrylic acid, 3-indoleethylamine hydrochloride, 3-indolepropionic acid, 3-indolelactic acid and tryptophan. The method depends upon ascending-descending paper partition chromatography employing isopropyl alcohol-ammonia-water as a solvent and 1 per cent *p*-dimethylaminobenzaldehyde in *N* HCl as the color reagent. A Densichron densitometer was employed to determine the optical density of the developed spots. When the logarithm of the concentration of indole compound is plotted against the per cent transmission of light a straight-line relationship ensues.

Ether extracts of soybean, tobacco, spinach, barley, and tomato leaf and stem tissues yielded negative tests for IAA. However, upon alkaline hydrolysis of barley and tobacco tissues IAA could be detected in the extracts. The view is expressed that "free" IAA was not present in these tissues, and that the bulk of the chemical was "bound" to a cellular component.²

² After the present manuscript had gone to press the following publication was received in this library: Yamaki, Toshio and Koshiro Nakamura. Formation of indoleacetic acid in maize embryo. Sci. Papers of the College of General Education, Univ. of Tokyo 2:

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81-98. 1952. These workers have utilized paper partition chromatography for the separation of 3-indoleacetic acid from other indole compounds in corn seed, and have shown that "bound" 3-indoleacetic acid in the endosperm of *Zea mays* is produced from proteins containing tryptophan in the course of alkaline hydrolysis.

STUDIES ON THE QUALITATIVE REQUIREMENTS OF
BLATTELLA GERMANICA (L.) FOR AMINO ACIDS
UNDER ASEPTIC CONDITIONS^{1,2}

JOHN D. HILCHEY

The German cockroach, *Blattella germanica* (L.) is widely used as an experimental subject. It has been found convenient for use not only in insecticidal screening, but also in studies on many phases of both intermediary metabolism and the mode of action of insecticides. In view of the fact that this species has shown the ability to develop resistance to the insecticide, chlordane, there is promise that it will also be useful in the investigation of resistance mechanisms. In order to provide proper control of experiments dealing with the intermediary metabolism of either normal or poisoned insects, it is essential to have an understanding of their basic nutritional requirements.

The general nitrogen requirements of *B. germanica* were investigated by Zabinski (23) who claimed that this species could subsist on a diet containing glycine as the only source of amino nitrogen. More recent studies by Melampy and Maynard (13) and McCay (12) have indicated that, since different proteins exhibit different nutritional values for *Blattella*, this insect does require certain specific amino acids in the diet. This view was supported by the research of Noland and Baumann (16) which demonstrated that the non-aseptic German roach requires both tryptophan and methionine for rapid growth. The most detailed studies of the amino acid requirements of *Blattella* were carried out by House (8) under aseptic conditions. He demonstrated that valine, tryptophan, histidine, and probably arginine were required for normal growth and that cystine was possibly required for normal development.

He also found that neither glycine nor methionine was required for normal growth or development.

The investigations described below were undertaken in order to evaluate the nutritional importance for *Blattella germanica* of those amino acids not investigated by House, namely, lysine, threonine, alanine, phenylalanine, tyrosine, leucine, isoleucine, proline, hydroxyproline, serine, aspartic acid, and glutamic acid.

¹ From a doctoral dissertation submitted to Cornell University, Ithaca, New York, September, 1951. The author gratefully acknowledges the guidance and assistance given him by his major advisor, Dr. Robert L. Patton, Insect Physiologist, Cornell University.

² This research was conducted at Cornell University, Ithaca, New York, with the aid of a grant from the Lalor Foundation of Wilmington, Delaware.

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MATERIALS AND METHODS

Except for minor adaptations, the apparatus used was that built by House (9) for his research. The rearing cage, transfer chamber, Salvioni balance, and equipment used to obtain asepsis were left basically unchanged. The anesthetizing device was altered only by the addition of a diaphragm valve to the carbon dioxide supply to permit easy regulation of the flow of gas.

The German cockroaches used for these studies were the progeny of females drawn at random from the Cornell-Rutgers stock used by House (7, 8, 9).

DIETS

The amino acid requirements of these insects were investigated using the diets listed below.

AACDA I, II, III, IV, V—Amino Acid Control Diet Aseptic. The control diets were made up according to the specifications given by House (7, Tables I and II).

Deficient diets. These were prepared according to the same formulations used for the AACDA diets except that individual amino acids were omitted. The total amino nitrogen level was maintained by replacing the loss due to the deletion of one amino acid with an equal weight of L-glutamic acid. When the dispensability of glutamic acid was under test, glycine was used to maintain the nitrogen level.

Special diets. "Super-cystine" and "Super-choline" diets were patterned after AACDA but contained twice the normal amount of cystine and choline respectively. "Sulfur-free" and "Methyl-free" diets were similar to AACDA but lacked cystine and methionine in the first instance and methionine and choline in the second. A "Separate-sterilization" diet was identical in content to AACDA but the amino acid mix was sterilized separately from the remaining dietary components and the two fractions were mixed in the transfer chamber.

In preparing these diets the dextrin, salts and crystalline amino acid mix were blended; the vitamins, sterols, soybean oil, and two milliliters of distilled water were mixed in the same manner. Then the two fractions were blended to uniformity in a mortar and allowed to dry; finally they were placed in 16-ounce widemouthed, paper-capped jars for sterilization. This was carried out by autoclaving the materials for 20 minutes at 5 pounds' pressure. The diets were then dried and introduced into the sterile chamber where they were ground to a fine powder before storage in the refrigerator.

The transfer case and Salvioni balance were sterilized by a thorough washing with 70 per cent ethanol. The other transfer equipment such as

diet spoons, spatulas, mortars, carbon dioxide dispensers, glass brei rods, and rubber hoses were autoclaved for 20 minutes at 15 pounds' pressure. The cage fittings and full water bottles were similarly treated and assembled.

In order to obtain aseptic nymphs, female cockroaches bearing ripe egg capsules were chosen at random from the stock culture. The capsules were removed and subjected to a sterilization process which consisted of washing for one minute in a 1:1 mixture of 0.2 per cent mercuric chloride and 95 per cent ethanol. The time of washing was reduced from that used by House³ in order to reduce excessive toxicity. The reduction in time produced no detectable increase in the rate of contamination of capsules and resulted in a marked increase in their viability. Furthermore, when washing time was decreased, fewer capsules had to be ruptured mechanically to permit emergence of the nymphs.

Before being used, all diets and capsules were checked for asepsis by inoculating slants of potato dextrose agar, proteose tryptone agar, and nutritive caseinate agar with breis of the material to be tested. Asepsis was considered attained if, after 72 hours of incubation at 37° C., no growth occurred on the media. In preliminary tests, the stab culture technique used by House (9) showed no advantage over the slant method and was discontinued.

ASSAY METHOD

After the diets and equipment had been prepared, the sterile roaches were placed on assay. The individuals from each capsule were chosen at random and distributed equally among all diets. All the roaches from the same capsule-diet group were placed in the same cage and held for 24 hours. Meanwhile an asepsis check was performed on the remains of the capsule plus any extra roaches. If, at the end of 24 hours, the remains of the capsule tested aseptic, the roaches from that capsule were placed in separate cages; they were allowed to continue on assay unless the 72-hour reading of the asepsis check showed contamination.

The routine of assay was established by House (9). The roaches were weighed each week for 12 weeks. The neck of a rearing cage was sterilized, placed in the access hole of the transfer chamber, and the cotton plug removed. A carbon dioxide dispenser was inserted to anesthetize the roach. After the animal had been removed from its cage, it was weighed and returned to the cage. The roaches were examined daily and the dates of moulting and maturity were recorded where possible. Since it was impossible to determine the sex of the nymphs at eclosion, the roaches were

³ House, Howard L. Personal communication.

held beyond the 12-week weighing period until they attained maturity. Any roaches that died during the assay were submitted to asepsis checks. At the end of the assay all roaches were sacrificed for such tests. The aseptic roaches which completed the assay, and for which sex data were either available or unnecessary, were used to evaluate the growth-supporting qualities of the diets. The data derived from these roaches were submitted to a statistical analysis (22). Finally the breis of several roaches were selected from each diet tested in the assays. An extract of each of these breis was analyzed qualitatively for free amino acids by the paper partition chromatographic methods outlined by Auclair (1) and Pratt (18). Certain diets could not be evaluated in this way because the material omitted from the diet was not clearly identifiable by these methods.

RESULTS AND DISCUSSION

The results of the statistical evaluation of the data derived from the nutritional assays are summarized in Tables I to VII and IX to XII. The data in Tables I to VII show that certain diets lacking specific amino acids produced rates of growth differing consistently and significantly from those produced by the corresponding control diets. The fact that a diet

TABLE I
GROWTH OF MALE *BLATTELLA GERMANICA* (L.) REARED ON DEFICIENT DIETS

Age of cock-roaches, in weeks	Mean weight and standard error (in milligrams) for diets:					
	AACDA III	Super-choline	-Lysine	-Threonine	-Phenyl-alanine	-Tyrosine
1	2.4 ± 0.06	2.1 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.05	2.3 ± 0.08
2	4.2 ± 0.2	3.7 ± 0.2*	3.6 ± 0.2**	3.9 ± 0.2	4.0 ± 0.1	4.3 ± 0.1
3	6.1 ± 0.5	5.9 ± 0.3	4.9 ± 0.4*	5.5 ± 0.4	6.0 ± 0.4	6.8 ± 0.3
4	7.8 ± 0.6	7.7 ± 0.4	6.8 ± 0.3	6.8 ± 0.3	7.8 ± 0.5	9.0 ± 0.7
5	11.4 ± 0.9	10.0 ± 0.5	10.0 ± 0.7	10.3 ± 0.7	10.2 ± 0.8	11.7 ± 0.8
6	14.6 ± 1.3	14.4 ± 0.9	13.6 ± 1.1	13.6 ± 1.3	15.2 ± 1.3	15.9 ± 1.5
7	17.5 ± 1.5	17.5 ± 1.0	17.1 ± 1.3	18.2 ± 2.2	17.9 ± 1.5	18.9 ± 2.2
8	22.9 ± 2.6	22.9 ± 1.6	22.0 ± 1.5	23.9 ± 3.1	25.7 ± 2.1	25.9 ± 2.7
9	32.4 ± 2.9	28.2 ± 1.7	27.2 ± 2.1	30.3 ± 4.0	30.5 ± 2.7	34.4 ± 3.8
10	34.2 ± 2.2	36.5 ± 2.4	34.9 ± 2.7	39.0 ± 4.1	38.9 ± 2.7	39.0 ± 2.8
11	42.0 ± 1.9	38.6 ± 2.0	38.9 ± 2.4	37.8 ± 3.9	41.4 ± 1.5	39.6 ± 2.5
12	42.9 ± 1.4	41.3 ± 1.8	41.5 ± 1.5	39.5 ± 3.0	45.4 ± 1.2	43.7 ± 2.3

* Difference from the control diet is significant at odds of 19:1.

** Difference from the control diet is significant at odds of 99:1.

lacking a specific amino acid supports growth only at a rate slower than that produced by the control shows clearly that this particular substance is required for growth. On the basis of this criterion it is evident from the data that, of those tested, the following amino acids are *essential* for the growth of German cockroaches of the specified sex:

TABLE II
GROWTH OF FEMALE BLATTELLA GERMANICA (L.) REARED ON DEFICIENT DIETS

Age of cock-roaches, in weeks	Mean weight and standard error (in milligrams) for diets:					
	AACDA III	Super-choline	-Lysine	-Threonine	-Phenyl-alanine	-Tyrosine
1	2.3±0.07	2.2±0.1	2.4±0.1	2.3±0.06	2.3±0.06	2.3±0.06
2	4.1±0.2	4.3±0.2	3.6±0.2	3.9±0.2	4.3±0.1	4.1±0.2
3	6.6±0.3	6.2±0.6	5.0±0.4**	5.7±0.3	6.5±0.3	6.1±0.5
4	8.5±0.5	7.7±0.7	6.9±0.4	7.3±0.4	8.7±0.5	8.5±0.8
5	11.0±0.6	10.8±0.7	9.1±0.6**	10.0±0.5*	12.0±0.5	11.0±0.8
6	16.9±0.6	13.6±1.6*	12.4±0.5**	15.2±1.0	16.3±1.2	15.5±1.1
7	19.3±1.5	17.5±1.1	16.6±1.5	19.9±1.7	21.5±1.6	20.7±1.8
8	27.6±2.1	22.6±1.5	21.1±1.3	26.7±2.3	27.4±2.4	28.1±2.3
9	36.5±2.7	32.3±2.5	29.3±1.3	38.2±3.9	39.9±3.4	39.2±3.1
10	48.0±3.6	41.0±3.7	37.5±2.6	49.5±3.9	47.0±4.1	51.4±4.0
11	56.1±2.9	48.5±5.3	48.7±4.1	60.3±4.7	57.3±3.4	63.0±3.1
12	64.6±3.4	59.4±3.9	60.2±3.4	66.2±4.0	66.9±3.1	71.8±3.0

* Difference from the control diet is significant at odds of 19:1.
** Difference from the control diet is significant at odds of 99:1.

TABLE III
GROWTH OF MALE BLATTELLA GERMANICA (L.) REARED ON DEFICIENT DIETS

Age of cock-roaches, in weeks	Mean weight and standard error (in milligrams) for diets:				
	AACDA IV	-Isoleucine	-Proline	-Hydroxy-proline	-Serine
1	2.1±0.1	1.8±0.1	2.0±0.1	2.0±0.1	1.9±0.1
2	3.2±0.2	2.4±0.3*	3.0±0.2	3.4±0.2	2.4±0.2*
3	4.7±0.3	3.2±0.3**	3.6±0.4**	4.4±0.3	3.3±0.2**
4	6.6±0.3	3.8±0.5**	5.6±0.3	6.5±0.4	5.3±0.4
5	9.0±0.6	5.2±0.5**	7.3±0.4*	8.7±0.7	6.6±0.6**
6	11.8±1.1	7.2±0.6**	8.7±0.5**	11.2±0.8	9.0±0.6**
7	14.6±1.1	8.9±0.8**	10.3±0.9**	14.5±1.1	10.6±0.9**
8	20.7±1.5	11.7±1.0**	11.4±0.9**	17.1±1.4*	12.3±0.9**
9	24.2±1.8	16.5±1.5**	15.6±1.2**	22.3±1.9	16.2±1.5**
10	31.8±2.8	20.2±2.3**	19.5±1.4**	28.1±2.2	19.4±1.8**
11	36.3±2.7	25.2±2.7**	26.2±2.0**	31.4±2.3	25.8±2.5**
12	39.5±1.7	29.9±1.9**	32.2±2.2*	38.7±2.1	31.2±2.6**

* Difference from the control diet is significant at odds of 19:1.
** Difference from the control diet is significant at odds of 99:1.

<i>Males</i>	<i>Females</i>
Alanine	Alanine
Serine	Lysine
Leucine	Leucine
Isoleucine	Isoleucine
Proline	
Lysine (possibly)	

It should be noted that these amino acids are in addition to those reported by House (8) as being required for normal growth of the German roach.

TABLE IV
GROWTH OF FEMALE *BLATTELLA GERMANICA* (L.) REARED ON DEFICIENT DIETS

Age of cock-roaches, in weeks	Mean weight and standard error (in milligrams) for diets:				
	AACDA IV	-Isoleucine	-Proline	-Hydroxyproline	-Serine
1	1.9±0.2	1.8±0.1	2.2±0.2	2.0±0.1	2.2±0.1
2	3.0±0.3	2.6±0.2	3.2±0.3	3.1±0.3	3.4±0.2
3	4.3±0.4	3.7±0.3	4.2±0.3	4.2±0.4	4.3±0.3
4	6.2±0.5	4.6±0.6*	6.3±0.5	5.7±0.4	6.4±0.3
5	8.1±0.5	6.7±0.8	7.0±0.6	7.8±0.7	9.0±0.7
6	11.2±0.7	8.8±0.9*	9.4±0.8	9.9±0.8	11.9±0.7
7	13.2±0.9	9.8±0.9*	11.6±1.2	11.3±2.8	14.0±1.0
8	17.4±1.5	13.6±1.4	14.0±1.0	13.0±1.3*	18.6±1.5
9	21.7±2.1	16.1±1.7	17.6±1.8	18.2±2.1	20.2±1.8
10	26.9±3.0	23.9±2.9	20.7±2.4	20.6±2.2	32.9±3.6
11	35.2±4.9	26.3±2.9	27.3±3.1	30.1±4.2	37.9±4.4
12	45.8±4.2	32.9±3.4*	36.8±3.8	35.3±4.7	47.9±4.0

* Difference from the control diet is significant at odds of 19:1.

It is also clear, on the same basis, that the following amino acids are *non-essential* for growth:

Males	Females
Threonine	Threonine
Phenylalanine	Phenylalanine
Tyrosine	Tyrosine
Hydroxyproline	Proline
Aspartic acid	Hydroxyproline
Glutamic acid	Serine
	Aspartic acid
	Glutamic acid

In two cases (males-lysine and females-isoleucine) the evidence, although convincing, is not as highly significant. Therefore, lysine may be qualified as being *possibly* essential for males, and isoleucine as being *probably* essential for females.

The results of the qualitative chromatographic analyses of tissue extracts of certain test roaches indicated that when alanine, serine, threonine, tyrosine, or proline was withheld from the diet, the amino acid withheld was found free in the tissue extract. When glutamic acid or aspartic acid was withheld, both the amino acid and the corresponding amide were found free in the tissue extract. When cystine and methionine were withheld simultaneously, large amounts of both were found in the tissue extract. Finally, when hydroxyproline was withheld, no evidence could be found of its presence in the extract; in fact, hydroxyproline is not normally found in the blood of this species even when provided liberally in the diet.

TABLE V
GROWTH OF MALE BLATTELLA GERMANICA (L.) REARED ON DEFICIENT DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:		
	AACDA V	-Aspartic acid	-Glutamic acid
1	2.1±0.1	2.1±0.1	1.9±0.2
2	3.4±0.2	3.5±0.2	3.3±0.2
3	4.5±0.3	4.6±0.3	4.7±0.3
4	7.0±0.2	7.5±0.2	7.3±0.3
5	9.4±0.5	10.2±0.6	11.3±0.7
6	11.3±0.5	13.1±0.7*	12.8±1.4
7	14.3±0.9	18.6±1.1**	18.5±1.2**
8	18.3±0.9	21.0±1.1	23.3±1.5**
9	21.5±1.6	30.3±1.7**	31.5±2.4**
10	25.7±1.4	35.3±1.6**	36.5±1.8**
11	36.6±2.3	39.9±1.7	38.8±2.0
12	36.9±1.7	40.5±1.2	41.8±0.9*

* Difference from the control diet is significant at odds of 19:1.

** Difference from the control diet is significant at odds of 99:1.

It was unnecessary to perform a statistical analysis in order to evaluate the performance of roaches fed diets lacking either leucine or alanine (Table VII). The differences between the diets and the controls were so large that the conclusions were obvious. Leucine and alanine are essential for the growth of both male and female *B. germanica*. While the results obtained by withholding leucine were anticipated, those obtained with alanine-free diets were quite unexpected.

The effects of the alanine deficiency were revealed in a complete departure of the test animals from the usual pattern of growth on deficient diets. In cases where other amino acids, such as isoleucine, proline, and

TABLE VI
GROWTH OF FEMALE BLATTELLA GERMANICA (L.) REARED ON DEFICIENT DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:		
	AACDA V	-Aspartic acid	-Glutamic acid
1	2.0±0.1	2.1±0.1	2.0±0.1
2	3.8±0.2	3.9±0.1	3.6±0.2
3	5.2±0.6	4.9±0.3	5.2±0.4
4	7.0±0.4	7.0±0.2	7.2±0.3
5	10.7±0.6	10.3±0.7	12.2±0.6
6	13.9±1.1	11.5±0.6	15.1±1.1
7	16.8±1.0	17.0±1.0	19.6±1.1
8	21.7±1.8	19.7±1.6	26.4±1.9
9	27.0±2.2	26.3±1.7	33.9±2.8*
10	35.1±3.4	31.6±2.4	41.1±3.4
11	45.3±4.1	42.5±3.7	53.4±3.9
12	53.0±4.4	51.2±4.4	59.7±3.7

* Difference from the control diet is significant at odds of 19:1.

serine, were withheld from diets, the roaches were able to maintain a moderate rate of growth despite the lack of a necessary nutrient. Although the time required for maturation was significantly increased, most such individuals eventually attained the adult stage. Moreover, a statistical analysis was performed on weight data taken at the time of maturity for roaches fed diets lacking either aspartic acid, glutamic acid, or organic sulfur. Results indicated that the weights of these roaches at maturity did not differ significantly from those of the control. In the case of the alanine-free

TABLE VII
GROWTH OF *BLATTELLA GERMANICA* (L.) REARED ON DEFICIENT DIETS,
AND THEIR RESPECTIVE CONTROL DIETS

Age of cockroaches, in weeks	Mean weight (in milligrams) for diets:						
	AACDA III	-Alanine I	AACDA IV	-Leucine	AACDA V	-Alanine II	Sulfur- free
1	2.3*	2.2	2.0*	1.8	2.0*	1.9	1.7
2	4.1	4.2	3.1	2.2	3.6	3.2	2.5
3	6.3	5.7	4.5	2.9	4.9	4.5	3.4
4	8.1	7.4	6.2	3.7	7.0	6.8	4.4
5	11.6	9.4	8.5	4.9	10.0	8.3	5.4
6	15.7	10.8	11.5	5.9	12.6	10.5	7.5
7	18.4	10.9	13.9	6.9	15.5	11.9	8.8
8	25.2	11.3	19.0	7.7	20.0	13.4	11.7
9	34.4	12.1	22.9	8.8	24.2	13.8	14.3
10	41.1	12.4	29.3	9.3	30.4	14.0	16.5
11	49.0	12.7	35.7	10.0	40.9	15.6	20.1
12	53.7	13.8	42.6	11.0	44.9	16.2	25.1

* An arbitrary mean of the performance of the controls, derived by calculating the mean of the mean performances of both male and female *Blattella germanica* (L.).

diets, the roaches stopped growing and survived as *third or fourth instar nymphs* for periods ranging from 9 to 47 weeks; all but one of these died without reaching maturity. When alanine, vitamins, or control diet were used to supplement or replace the alanine-free diet after 12 weeks of deprivation, the roaches failed to return to the normal growth pattern. This is interpreted to indicate that the effects of the alanine deficiency are irreversible.

As a check on these findings, another alanine-free diet was prepared and assayed. The results were essentially the same as in the case of the first diet. Chromatographic analyses of the roaches fed the second lot of diet showed that alanine was synthesized in relatively large quantities by the German roach. There appears to be no doubt that despite their ability to synthesize this substance, the roaches required alanine *under the conditions of these experiments*. The indispensability of alanine for *Blattella* is unusual in view of the fact that there are no records indicating a requirement for this amino acid in other animals. It is possible that the effects

produced in such a deficiency may not be direct, but may be due to the failure of some detoxifying or sparing action normally performed by alanine when provided in sufficient amounts under these experimental conditions. No data exist at present to support any hypothesis explaining this phenomenon which requires further investigation.

The performance of the cockroaches fed the leucine-free diet was the only instance, other than those of the alanine and labile methyl-free diet, in which the growth pattern of the test animals was radically different from the norm. As was the case in the alanine assays, growth ceased after the first few weeks of the test. Unfortunately, no information could be obtained on the survival of roaches fed a diet lacking leucine.

The feeding of glutamic and aspartic acids produced a unique response in the insects. The data in Table V show that not only are these two amino acids dispensable but they actually retard the growth of male *B. germanica*. This effect is similar to that observed in *Aedes aegypti* (L.) larvae (4). However, it should be noted that only male German roaches showed this toxic reaction; the females were apparently able to cope with these substances when fed at the standard level (Table VI). Since these two amino acids are structurally unique among the naturally-occurring forms, it is possible that the explanation for these biological reactions may be found in the chemical structure common to both of these compounds.

In view of the apparent toxicity of glutamic acid for male *Blattella*, a question arises as to whether the substitution of glutamic acid for an amino acid deleted from the diet seriously biased the assays. There is no significant correlation between the amount of glutamic acid present in the diet and the degree of suppression of insect growth on deficient diets. Therefore, this technique has not unduly influenced the results of these assays. The substitution of glycine for glutamic acid in the assay of the glutamic acid-free diet produced no discrepancy. Glycine is not essential for growth (8) and, therefore, could not have produced the significant differences reported for this diet.

Other sexual differences occurred in response to the feeding of deficient diets. Male German roaches require the presence of both serine and proline in the diet, while females do not. On the other hand, female roaches are apparently much more sensitive to lysine deficiency than are males. While no specific explanation can be given for these anomalies, it is clear that innate sexual differences in the mode or rate of metabolism of these amino acids are responsible.

The amino acid requirements of the German cockroach seem to differ radically from those known for other insects and for the rat. Of the ten most generally required, only seven are needed by *Blattella* (Table VIII) for growth. All other species listed in this table require a dietary source of phenylalanine; with this material as a substrate, they can satisfy their

metabolic needs for the closely related tyrosine. This synthesis cannot be reversed. *Blattella*, however, grows equally well when either tyrosine or phenylalanine is fed. It appears that the two may be interconvertible and therefore metabolically equivalent. A similar situation seems to exist in the case of the sulfur-containing amino acids, cystine and methionine. House (8) was unable to detect free methionine in the blood of German roaches deprived of a dietary source of this substance; neither was he able

TABLE VIII

AMINO ACID NUTRITIONAL REQUIREMENTS FOR GROWTH OF SEVERAL SPECIES OF ANIMALS

Amino acid	Animal species					
	<i>Blattella germanica</i> (8)	<i>Drosophila melanogaster</i> (6, 10, 20)	<i>Aedes aegypti</i> (4)	<i>Atta-genus</i> (?) sp. (14)	<i>Tribolium confusum</i> (11)	Rat (19)
Glycine	—*	+	+	—	—	—
Alanine	M, F	—	—	—	—	—
Valine	+	+	?	+	+	+
Leucine	M, F	+	+	+	+	+
Isoleucine	M, F	+	+	+	+	+
Serine	M (only)	—	—	—	—	—
Threonine	—	+	+	+	+	+
Phenylalanine	—	+	+	+	+	+
Tyrosine	—	—	—	—	—	—
Methionine	—*	+	+	+	+	+
Cystine	—*	—	—	—	—	—
Aspartic acid	—	—	—	—	—	—
Glutamic acid	—	—	—	—	—	—
Lysine	M, F	+	+	+	+	+
Arginine	Probably*	+	+	+	+	+
Histidine	+	+	+	+	+	+
Proline	M (only)	—	—	—	—	—
Hydroxyproline	—	—	—	—	—	—
Tryptophan	+	+	+	+	+	+

M = Required by male.

F = Required by female.

+ = Required, presumably, by both sexes.

— = Not required, presumably, by either sex.

? = Requirement not known.

* These amino acids assayed by House (8) who made no distinction between sexes.

to detect free cystine under similar circumstances. Evidence was also obtained that a specific dietary requirement for cystine must be satisfied in order for normal development to take place. House found that nymphs lacking a dietary source often died in the process of moulting. However, the fact that the growth appeared normal as compared to the control even when one or the other of these amino acids was omitted from the diet indicates that there may have been some interconversion.

Noland and Baumann (16) found that methionine was required by *Blattella* for optimum growth under non-aseptic conditions. They also

attempted to determine whether the synthesis of methionine which they observed was the result of action on the part of the gut flora or due to the intrinsic ability of the roach. In these studies they fed the roaches a methionine-free diet containing 1 per cent succinylsulfathiazole. The roaches grew very poorly under these conditions; the authors therefore concluded that the synthesis was carried out by the flora of the gut. It is quite possible that this technique not only eliminated the bacteria from the gut, but also interfered seriously with the normal metabolism of the roach, effectively precluding the possibility of synthesis by the cockroach itself.

In order to aid the clarification of such relationships, a diet lacking both cystine and methionine and a diet lacking both methionine and choline were assayed. The data in Table VII show clearly that the diet lacking organic sulfur could support only a slow rate of growth. However, no evidence of the moulting failure described by House (8) could be found. Further work will be necessary before this discrepancy can be explained. It is clear that a source of organic sulfur is required in order for normal growth to occur. Nevertheless, evidence from the chromatographic analysis of tissue extracts proved that the German roaches fed this diet had synthesized moderate amounts of both cystine and methionine. The only source of sulfur in the diet in large enough quantities to permit such synthesis was the sodium sulfate provided in the salt mix. Therefore, the sulfate ion must have been reduced to form the sulphydryl radical prior to the incorporation of the sulfur into amino acids.

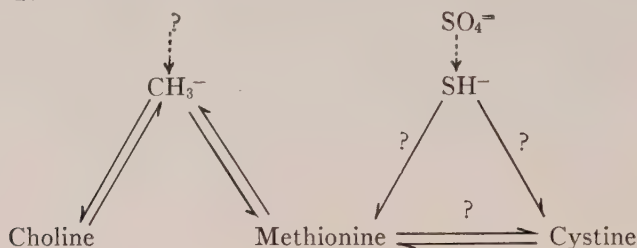
The results obtained with a diet lacking both cystine and methionine show that synthesis of these substances by the roach itself does take place. This does not answer the question of whether the interconversion of these two amino acids can occur. In the absence of a dietary source of one of these amino acids, the metabolic needs for this material may have been supplied either by independent synthesis, by interconversion, or by a combination of both. The studies of Noland and Baumann must also be considered. Therefore, it appears the cystine and methionine may be supplied to the German cockroach by means of one or a combination of the following routes: (a) through dietary supply; (b) as the result of synthesis by the gut flora; or (c) as the result of synthesis on the part of the roach itself or its "intracellular symbionts." The relative importance of any single route or combination of routes will depend largely upon the nature of the conditions under which the roach is reared, i.e., in a normal septic environment, under aseptic conditions, in the presence of antibiotics, and so forth.

Only 10 per cent of the roaches survived and matured on a diet lacking labile methyl donors; the remainder perished early in the assay period. It is clear, therefore, that the German cockroach has a strict dietary requirement for sources of labile methyl groups. These results are quite in agree-

ment with those obtained by using contaminated roaches (16). However, chromatographic analysis of the surviving roaches indicated that methionine was present in the tissue extracts. These individuals obviously had synthesized labile methyl groups even though others may have been incapable. Results released since the time at which the present work was done indicate that the rat is also able to synthesize this important chemical grouping (3).

A tentative schematic representation of the metabolic relationships of the ~~sulfur amino~~ acids may now be erected:

Sulfur amino



In the future it should be possible to examine these hypothetical relationships more closely in an effort to establish the validity of these hypotheses or to determine the nature of needed revisions.

The absence of a requirement for threonine in this insect is inexplicable on the basis of present knowledge. The results of the chromatographic analyses indicate that threonine is present in the blood of German roaches even when this amino acid is withheld from the diet. Thus it can only be said that threonine can be synthesized at a rate sufficient for growth.

Other amino acids can be synthesized by *Blattella*. It has been shown that most of the dispensable amino acids can be synthesized when they are withheld from the diet. Hydroxyproline could not be detected either in the roaches fed the control diet or in those fed the deficient diet. Phenylalanine, lysine, leucine, and isoleucine could not be unequivocally detected by the methods used. Therefore, it is not possible to decide whether these dispensable nutrients can be synthesized. However, there is good evidence that, despite the fact that alanine, proline, and serine are required for growth, these amino acids are synthesized. Even when these compounds are withheld from the diet, large amounts may be found in tissue extracts of *Blattella*. Therefore, these amino acids are synthesized but not at a rate commensurate with growth.

LIMITING EFFECTS

It is possible to evaluate the limiting effects of certain phenomena occurring during these assays in the light of the recorded experiences of workers who have dealt with similar problems. House (7) observed in his

work that the diet, SDNA⁴ permitted a higher rate of growth than did the aseptic amino acid control diet. In addition, it was noted, in the course of the present work, that in almost all cases the cockroaches fed diet AACDA passed through a minimum of seven moults. On the other hand, roaches reared in stock colonies on SDNA nearly all passed into maturity at the sixth moult. According to Seamans and Woodruff (21), the interpolation of an extra moult into the life cycle of *Blattella germanica* is a clear sign of nutritional deficiency.

The data which would permit a discussion of the relative nutritional value of all components of these diets are not available. However, three points of doubt may be eliminated. The minimum requirement of *B. germanica* for choline has been designated as 2000 micrograms per gram of diet (15). The standard level of choline used for the present assays was

TABLE IX
GROWTH OF MALE BLATTELLA GERMANICA (L.) REARED ON SPECIAL DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:			
	AACDA I	AACDA II	Super-cystine	Separate-sterilization
1	2.1 ± 0.1	2.5 ± 0.3	2.6 ± 0.2	2.5 ± 0.2
2	3.6 ± 0.4	4.0 ± 0.3	3.7 ± 0.3	3.6 ± 0.3
3	4.7 ± 0.7	5.9 ± 0.7	5.0 ± 0.6	5.5 ± 0.7
4	7.6 ± 1.1	8.1 ± 0.9	7.0 ± 0.8	8.7 ± 0.8
5	10.6 ± 0.9	13.7 ± 0.8	9.7 ± 1.3	10.9 ± 0.9
6	12.7 ± 2.1	15.1 ± 1.6	12.5 ± 1.6	15.0 ± 1.5
7	18.5 ± 1.6	21.2 ± 2.1	17.2 ± 2.0	19.8 ± 2.7
8	23.8 ± 4.7	25.8 ± 2.5	22.7 ± 2.0	24.8 ± 2.3
9	29.7 ± 3.0	33.9 ± 4.4	29.2 ± 2.8	33.9 ± 4.4
10	38.0 ± 3.2	37.8 ± 2.9	32.3 ± 2.7	41.1 ± 3.2
11	42.7 ± 1.7	41.0 ± 3.4	43.7 ± 1.7	45.9 ± 3.2
12	45.2 ± 1.9	45.3 ± 1.4	44.3 ± 1.1	44.1 ± 2.3

1000 micrograms per gram of diet. The results of assaying a diet containing the recommended amount of choline (diet, Super-choline, Tables I and II) indicated that the level of choline used in the present assays was not a limiting factor. It was also suspected that the cystine content of the control diets might be too low to permit optimum growth. However, doubling the cystine content of the control diet did not increase its nutritional value (diet, Super-cystine, Tables IX and X). Therefore, it may be presumed that the standard level of cystine used in these assays was not a limiting factor. A third possibility was that autoclaving certain amino acids of the diet in the presence of dextrin might promote the destruction of those amino acids (17). Autoclaving the amino acid mixture separately from the other dietary components produced no significant improvement in the control diet (diet, Separate-sterilization, Tables IX and X).

Other effects may operate to lower the nutritional efficiency of the

⁴ Non-aseptic stock diet; dried skimmed milk, whole wheat flour, and dried brewer's yeast in the proportions, 2:2:1.

control diets. Some amino acids, such as glutamic and aspartic acids, may be toxic at certain concentrations in the diet (4). Moreover, it is known that, while some unnatural forms of amino acids may be partially metabolized (19), others may actually inhibit the utilization of the corresponding L forms (24). Finally, it has been shown that the utilization of one amino acid may depend on the relative concentrations of other amino acids present in the diet (2). Thus the degree of approach to optimum utilization must depend, ultimately, upon the degree of approach to the

TABLE X
GROWTH OF FEMALE *BLATTELLA GERMANICA* (L.) REARED ON SPECIAL DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:			
	AACDA I	AACDA II	Super-cystine	Separate- sterilization
1	3.0 ± 0.3	2.7 ± 0.3	2.5 ± 0.2	2.7 ± 0.2
2	4.1 ± 0.2	4.3 ± 0.1	3.7 ± 0.3	3.7 ± 0.3
3	6.1 ± 0.7	5.5 ± 0.5	5.4 ± 1.0	5.7 ± 0.5
4	8.6 ± 1.1	8.6 ± 0.7	7.9 ± 1.0	9.9 ± 0.9
5	11.8 ± 0.7	13.4 ± 1.3	12.4 ± 1.5	13.8 ± 0.8
6	15.1 ± 1.4	16.7 ± 1.3	16.5 ± 1.5	16.6 ± 1.3
7	19.6 ± 1.8	20.2 ± 2.2	23.1 ± 3.3	23.9 ± 2.6
8	24.8 ± 2.5	26.4 ± 2.1	30.9 ± 2.7	28.3 ± 2.5
9	32.8 ± 3.1	34.7 ± 4.6	41.4 ± 4.4	34.6 ± 1.7
10	39.8 ± 4.2	46.2 ± 3.9	52.4 ± 5.9	52.1 ± 4.8
11	54.4 ± 3.9	56.0 ± 5.1	62.4 ± 5.7	61.8 ± 3.9
12	60.7 ± 3.9	72.8 ± 4.0	73.1 ± 5.9	73.1 ± 4.0

optimum dynamic balance of the remaining amino acids and other nutrients. The detection, measurement, and adjustment of these effects are beyond the scope of the present research. The work at hand forms the foundations upon which studies of interrelations may be carried out. Until these relationships are understood, care must be exercised in the evaluation of the nutritional qualities of test diets.

SOURCES OF VARIATION AFFECTING STATISTICAL ANALYSIS

Variation due to sex. Variations in the ratios of males to females fed various diets may affect the results of statistical analysis (5). In order to eliminate these effects, the data from males and females were analyzed separately.

Capsule variation. A number of egg capsules were used to provide enough roaches for each assay. Each capsule was divided equally among all diets. However, deaths, contaminations, and the variability of the sex ratio within individual egg capsules produced differences in the number of roaches in any diet-capsule unit. The cockroaches from different capsules

TABLE XI

VARIATION IN GROWTH OF MALE BLATTELLA GERMANICA (L.) REARED ON CONTROL DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:		
	AACDA III	AACDA IV	AACDA V
1	2.4 ± 0.06	2.1 ± 0.1*	2.1 ± 0.1*
2	4.2 ± 0.2	3.2 ± 0.2**	3.4 ± 0.2**
3	6.1 ± 0.5	4.7 ± 0.3**	4.5 ± 0.3**
4	7.8 ± 0.6	6.6 ± 0.3	7.0 ± 0.2
5	11.4 ± 0.9	9.0 ± 0.6	9.4 ± 0.5
6	14.6 ± 1.3	11.8 ± 1.1*	11.3 ± 0.5*
7	17.5 ± 1.5	14.6 ± 1.1	14.3 ± 0.9
8	22.9 ± 2.6	20.7 ± 1.5	18.3 ± 0.9
9	32.4 ± 2.0	24.2 ± 1.8**	21.5 ± 1.6**
10	34.2 ± 2.2	31.8 ± 2.8	25.7 ± 1.4**
11	42.0 ± 1.9	36.3 ± 2.7	36.6 ± 2.3
12	42.9 ± 1.4	39.5 ± 1.7	36.9 ± 1.7

* Difference from AACDA III is significant by odds of 19:1.

** Difference from AACDA III is significant by odds of 99:1.

are known to vary in vigor and, therefore, in their rate of growth. If these differences in performance were significant, the disproportionate numbers of individuals in the diet-capsule units could cause bias in the statistical analysis. A preliminary analysis showed that the variations in vigor of the roaches from different capsules were due to chance alone. Therefore, a simple analysis of variance could be used in evaluating the performance of cockroaches used for nutritional assays (22).

Variation in controls. Differences were observed between the mean weights of the groups of roaches fed control diets in three successive assays. An analysis of variance showed that these differences were significant

TABLE XII

VARIATION IN GROWTH OF FEMALE BLATTELLA GERMANICA (L.) REARED ON CONTROL DIETS

Age of cockroaches, in weeks	Mean weight and standard error (in milligrams) for diets:		
	AACDA III	AACDA IV	AACDA V
1	2.3 ± 0.07	1.9 ± 0.2*	2.0 ± 0.1
2	4.1 ± 0.2	3.0 ± 0.3**	3.8 ± 0.2
3	6.6 ± 0.3	4.3 ± 0.4**	5.2 ± 0.6**
4	8.5 ± 0.5	6.2 ± 0.5**	7.0 ± 0.4*
5	11.9 ± 0.6	8.1 ± 0.5**	10.7 ± 0.6
6	16.9 ± 0.6	11.2 ± 0.7**	13.9 ± 1.1*
7	19.3 ± 1.5	13.2 ± 0.9**	16.8 ± 1.0
8	27.6 ± 2.1	17.4 ± 1.5**	21.7 ± 1.8*
9	36.5 ± 2.7	21.7 ± 2.1**	27.0 ± 2.2**
10	48.0 ± 3.6	26.9 ± 3.0**	35.1 ± 3.4*
11	56.1 ± 2.9	35.2 ± 4.9**	45.3 ± 4.1
12	64.6 ± 3.4	45.8 ± 4.2**	53.0 ± 4.4*

* Difference from AACDA III is significant at odds of 19:1.

** Difference from AACDA III is significant at odds of 99:1.

(Tables XI and XII). Since the control diets were made as nearly identical as was practicable, and since the egg capsules were chosen at random from the colony, these differences must represent variations in the general level of vigor of the stock colonies. These variations create no obstacle to the assay or to the analysis of data obtained from the assays as long as a suitable control diet is assayed with the deficient diets.

SUMMARY

1. The dietary requirements of German cockroaches, *Blattella germanica* (L.), for the amino acids alanine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, aspartic acid, glutamic acid, lysine, proline, and hydroxyproline were investigated under aseptic conditions. The results, which supplement the work of House, indicate that the requirements differ for the two sexes.

2. Of the amino acids tested in the present experiments, males required alanine, leucine, serine, isoleucine, proline, and possibly lysine; females required alanine, leucine, lysine, and probably isoleucine.

3. The nutritional and metabolic significance of certain of these substances were discussed. The results of the experiments indicate that the dietary requirements of German roaches for amino acids may differ considerably from those known for other insects.

4. The available evidence indicates that the German cockroach is not only able to synthesize biologically labile methyl groups, but also can transform inorganic sulfur (SO_4^-) into an organic form (SH^-) for incorporation into cystine and methionine.

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STUDIES ON THE NATURE OF FUNGICIDAL ACTION. I. INHIBITION OF SULFHYDRYL-, AMINO-, IRON-, AND COPPER-DEPENDENT ENZYMES IN VITRO BY FUNGICIDES AND RELATED COMPOUNDS

ROBERT G. OWENS¹

INTRODUCTION

Adequate data exist to indicate that enzyme inhibition is one mechanism involved in the control of disease agents with chemicals (3, 4, 5, 6, 7, 11, 12, 13, 15, 17, 21, 22, 29, 30, 31, 32, 34, 35, 37, 41, 42, 43, 44, 45, 50, 51, 52, 53, 54, 55). However, the extent to which different enzymes may interact with the same chemical, the relative contributions of these interactions to growth effects, or the physicochemical factors underlying inhibition are largely unknown in the case of fungicides. Since these factors occupy a basic position in fungicidal action mechanisms, studies have been undertaken towards their elucidation.

In the present study the problem is approached from the standpoint of the relative sensitivity of various types of enzymes to chemicals and the chemistry of enzyme-chemical interactions. It involves a survey of various classes of chemicals and their effects on the catalytic activity of several major enzyme types. The enzymes, in turn, are used as assay agents to indicate the relative reactivity of different chemicals with catalytically active groups of enzyme types occurring in all biological systems. Since many of the properties of the chemicals are known, inhibition data are used further to indicate relationships between these properties, chemical constitution and biological action mechanisms. The enzymes used are regarded as representative of enzyme types and are not assumed to be the specific enzymes involved in fungicidal mechanisms. Interactions of these enzymes with the chemicals are regarded, therefore, only as potential interactions that may occur in living systems. Ultimate elucidation of precise *in vivo* mechanisms must involve knowledge of potential reactions with all types of enzymes and the relative sensitivity of specific enzymes to specific chemical agents.

This paper reports results from studies on pancreatic amylase, malt amylase, catalase, and polyphenol oxidase, which represent, respectively, amino-, sulfhydryl-, iron-, and copper-dependent enzymes (2, 7, 9, 10, 19, 20, 23, 26, 27, 29, 36, 39, 40, 43, 44, 45, 47, 48, 49). Inhibition of these enzymes by Hg^{++} , Cu^{++} , Zn^{++} , Fe^{+++} , benzoquinones, naphthoquinones,

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phenols, 8-hydroxyquinolines, dithiocarbamates, phenylmercuric acetate, and elemental sulfur is compared and discussed with respect to active enzyme groups and some known properties of the chemicals. The nature of chemical properties related to chemical-enzyme interactions and some effects of chemical constitution and substitution as indicated by inhibition data are discussed. Some brief observations are made concerning probable chemical-enzyme interaction mechanisms and some relationships between *in vitro* action and *in vivo* toxicity of fungicides.

MATERIALS AND METHODS

GENERAL

Test chemicals. Active components of fungicides were supplied by fungicide manufacturers in at least 95 per cent pure form.² Other test chemicals were C.P., U.S.P., or the best Eastman Kodak Company grades. Chemicals were dissolved in absolute ethyl alcohol (not more than 2 per cent of the final volume) with gentle warming and then brought to the desired dilution with water. Addition of water to alcoholic solutions of sulfur, ferric dimethyldithiocarbamate, zinc dimethyldithiocarbamate, tetramethylthiuram disulfide, 2,3-dichloro-1,4-naphthoquinone, tetrachloro-*p*-benzoquinone, phenylmercuric acetate, copper-zinc-chromate complex, or 5,7-dichloro-8-hydroxyquinoline resulted in insoluble colloidal or crystalline material at a concentration of 10^{-3} *M*. In some cases undissolved material persisted even at concentrations at which inhibition determinations were made. Data on inhibition in these cases are considered only as qualitative indications of interaction.

Stock buffers. The hydrogen ion concentration of solutions was regulated by use of 0.3 *M* acetate or phosphate buffer. Monobasic potassium phosphate and dibasic sodium phosphate were combined to give a pH of 7.0, 6.8, and 6.8 for pancreatic amylase, catalase, and polyphenol oxidase systems, respectively, after dilution in reaction flasks. Acetic acid and sodium acetate were combined to give a pH of 4.6 for malt amylase systems. For activation of pancreatic amylase 0.065 *M* sodium chloride was included in the stock buffer.

PREPARATION OF ENZYME SYSTEMS AND TESTS FOR INHIBITION

Amylases. Preparations containing either pancreatic or malt amylase were obtained from Nutritional Biochemicals Corporation, Cleveland,

² The author is indebted to the Naugatuck Chemicals Division of U. S. Rubber Company for samples of 2,3-dichloro-1,4-naphthoquinone and tetrachloro-*p*-benzoquinone, to E. I. du Pont de Nemours & Company for samples of dithiocarbamates, to Union Carbide and Carbon Corporation for samples of copper-zinc-chromate complex, to Benzol Products Company for a sample of oxyquinoline base, and to California Spray-Chemical Corporation for a sample of phenylmercuric acetate.

Ohio. One ml. of a solution containing 0.5 mg./ml. of 2X U.S.P. pancreatin was used in each reaction flask for pancreatic amylase inhibition determinations whereas 1 ml. of a solution containing 2.5 mg./ml. of analytical grade malt amylase preparation was used for malt amylase inhibition determinations. These quantities of the two preparations liberated reducing groups from starch at approximately the same rate under these test conditions.

Mallinckrodt soluble starch was used as the substrate for both amylases. A suspension of 25 grams starch in 150 ml. of water at room temperature was added slowly with stirring to 850 ml. of boiling water in a 2-liter Erlenmeyer flask. The solution was boiled for three minutes after addition was completed, transferred to a 1-liter volumetric flask, cooled to the hydrolysis temperature, and then made up to volume. The flask was then immersed in a water bath at 37° C. or 40° C. for pancreatic and malt amylase, respectively.

Nine ml. of the appropriate buffer or buffer-sodium chloride solution were added to 50-ml. Erlenmeyer flasks from a burette. Twenty ml. of test chemical solutions were then added from a 20-ml. volumetric pipette. The flasks were stoppered with waxed corks and placed in a water bath at 37° C. or 40° C., depending on the amylase under test. One ml. of enzyme solution, diluted as described above, was added to each successive flask at one-half minute intervals. The enzyme and test chemical were incubated together for two hours, and then 10 ml. of starch solution were added to each successive flask at one-half minute intervals in the same order that enzyme was added. At exactly 15 minutes after addition of starch to each flask, 3 ml. of 1 *N* hydrochloric acid were added to stop enzyme activity.

Activity of amylase was determined by a modification of the ferricyanide titration method recommended by the American Society of Brewing Chemists and described by Tauber (45, pp. 84-85). Five ml. of hydrolysate were transferred from digestion flasks to 125-ml. Erlenmeyer flasks. Ten ml. of alkaline ferricyanide were added and the solutions autoclaved at 15 p.s.i. for 15 minutes. After cooling, 25 ml. of potassium chloride-zinc sulfate-acetic acid reagent and 1 ml. of 50 per cent potassium iodide solution were added. The liberated iodine was then titrated with 0.05 *N* sodium thiosulfate with starch as the indicator. Activity determinations were corrected for reactions between reagents and test chemicals and for the reducing groups present in unhydrolyzed starch.

Catalase. The method of Jolles as described by Sumner and Somers (44, pp. 209-210), for catalase activity determinations was followed except for modifications to permit incubation of test chemicals with the enzyme. A preparation containing 4320 Keil units of catalase per gram was obtained from Armour and Company. One ml. of a solution containing 0.001 mg./ml. was used in each reaction flask. Test systems contained 9 ml.

of phosphate buffer, pH 6.8, in 50-ml. Erlenmeyer flasks. Twenty ml. of test chemical solution were added and incubated with 1 ml. of enzyme solution for one and one-half hours at room temperature. The flasks were then placed into a cracked-ice bath. After an additional one-half hour of incubation at 2° to 3° C., 10 ml. of 0.6 per cent hydrogen peroxide, previously cooled in the ice bath, were added as described for addition of starch to amylase systems.

Catalase activity was allowed to continue for 20 minutes and then 5 ml. aliquots were transferred to 125-ml. Erlenmeyer flasks containing 5 ml. of 2 *N* sulfuric acid. To this was added 2 ml. of 50 per cent potassium iodide solution and one drop of 1 per cent ammonium molybdate solution. The liberated iodine was titrated with 0.01 *N* sodium thiosulfate with addition of starch as an indicator. Activity was based on the disappearance of hydrogen peroxide.

Polyphenol oxidase (tyrosinase). A preparation of polyphenol oxidase was obtained by extraction of the commercial mushroom, *Agaricus campestris* L., as described by Tenenbaum and Jensen (46). The extract was stored under refrigeration after the second fractionation of the original extract. This was diluted 1:5 for use. Activity determinations were based on the method described by Adams and Nelson (1).

One ml. of phosphate buffer, pH 6.8, was put into the body of a Warburg flask. Two ml. of test chemical solution and 0.5 ml. of polyphenol oxidase solution were added. Five-tenths ml. of a 2 per cent solution of pyrocatechol was pipetted into the side arm. The flasks were attached to the manometers, placed in the water bath, and incubated with shaking for two hours at 30° C. After the incubation period the pyrocatechol solution was mixed with the enzyme and test chemical in the body of the flasks, and shaking was resumed, at 100 strokes per minute. Oxygen uptake was recorded at 5-minute intervals over a 30-minute period. Inhibition percentages were corrected for changes in barometric pressure and for air-oxidation of pyrocatechol.

RESULTS

TEST CHEMICAL CONCENTRATIONS

Preliminary tests showed that some compounds stimulated enzyme activity when present in low concentrations but inhibited in higher concentrations. Therefore, inhibition by all compounds was compared to that by mercuric chloride which, at 10^{-5} *M*, inhibited both amylases essentially 100 per cent. To allow for somewhat less inhibitory compounds, which might nevertheless be moderately potent inhibitors, 10^{-3} and 10^{-4} *M* were selected arbitrarily for initial tests. The limited solubility and the fact that some materials interfered seriously with activity determinations discouraged testing at higher concentrations. Since none of

the chemicals inhibited polyphenol oxidase completely at 10^{-3} *M*, they were not tested at 10^{-4} *M* against this enzyme.

EFFECTS OF INCUBATION OF CHEMICALS WITH THE ENZYMES

Data in Table I show that incubation of mercuric chloride, copper chloride, *p*-benzoquinone, or 1,4-naphthoquinone with pancreatic or malt amylases for two hours increased inhibition, but the effects of incubation were much more pronounced in case of *p*-benzoquinone and 1,4-naphthoquinone than in case of mercuric chloride and copper chloride.

TABLE I
COMPARATIVE EFFECTS OF INCUBATION ON INHIBITION OF PANCREATIC AND MALT
AMYLASES BY METAL IONS AND QUINONES

Chemical (10^{-4} <i>M</i>)	Inhibition, per cent			
	Pancreatic amylase		Malt amylase	
	Incubation 2 hours	No incubation	Incubation 2 hours	No incubation
Mercuric chloride	100	86	97	95
Copper chloride	48	11	55	30
<i>p</i> -Benzoquinone	93	0	94	4
1,4-Naphthoquinone	24	0	93	0

In another experiment, Figure 1, pancreatic amylase inhibition by 1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone was determined as a function of incubation time. Inhibition by 2,3-dichloro-1,4-naphthoquinone increased at a more rapid rate than that by 1,4-naphthoquinone. Differences in inhibition increased with incubation time so that small, indeterminate differences obtained with short incubation periods became significantly large as the incubation period was increased. The increased differences thus obtained were of value in the present study because they increased the relative sensitivity of assay methods and aided in analyzing specific chemical properties which regulate chemical-enzyme interactions.

COMPARATIVE INHIBITION OF THE ENZYMES BY VARIOUS CHEMICALS

Interactions of the various chemicals with pancreatic and malt amylases, catalase, and polyphenol oxidase can be compared from inhibition data presented in Table II. For convenience of discussion the chemicals were divided into five groups on the basis of similarities in molecular structure or substituents. Such groupings were found to aid in relating chemical structure and properties to enzyme inhibition.

Elemental sulfur, Group I, was singular in that it inhibited none of the enzymes strongly, probably because of its extremely low solubility in water.

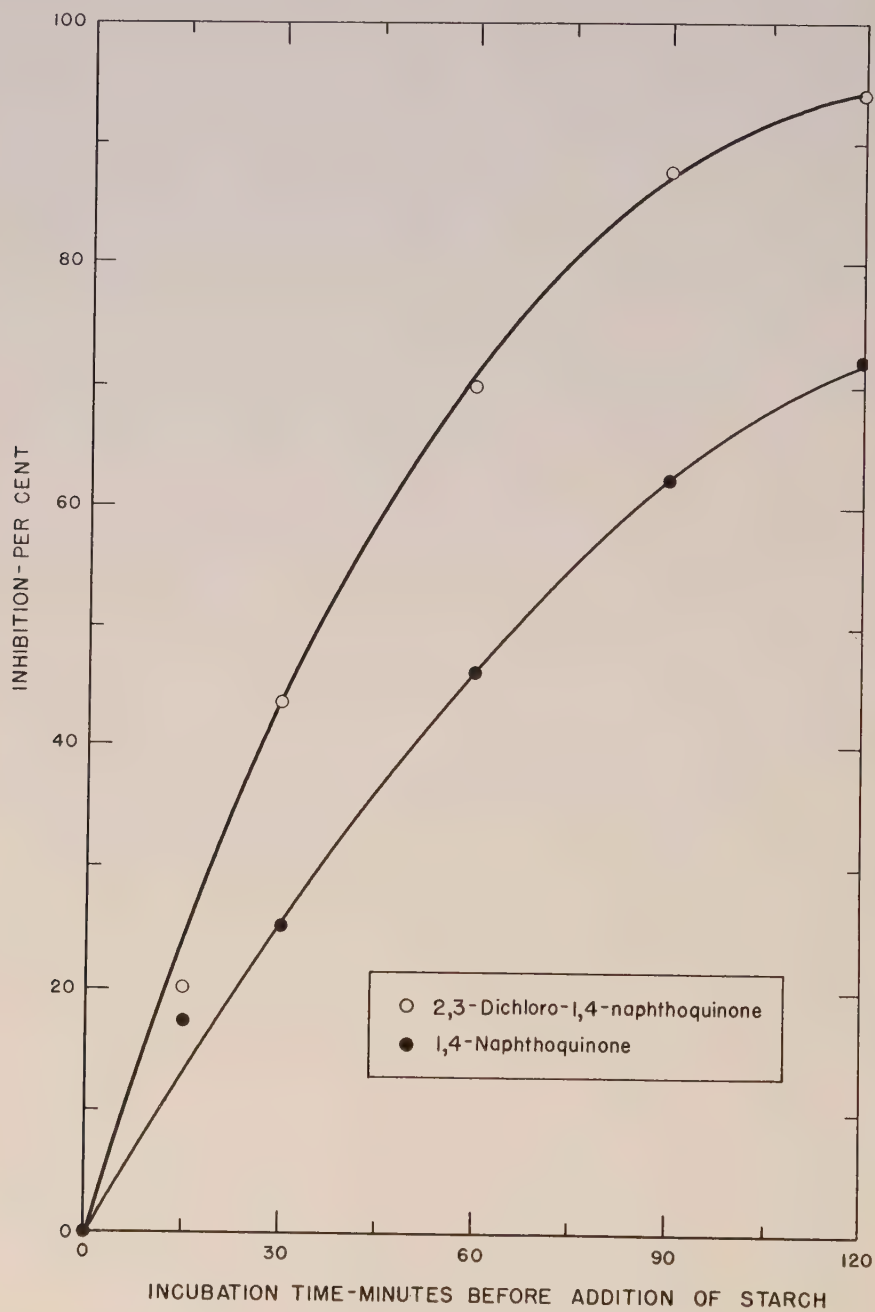


FIGURE 1. Effect of incubation time on inhibition of pancreatic amylase by quinonoid compounds.

However, on various occasions it inhibited all enzymes slightly. This inhibition may be due to oxidation products of sulfur rather than to sulfur itself.

Dithiocarbamic acid derivatives, Group II, interacted with all enzymes tested. Sodium diethyldithiocarbamate reduced the activity of polyphenol oxidase 79 per cent, but inhibited catalase and amylases less than 25 per cent. Ferric dimethyldithiocarbamate, zinc dimethyldithiocarbamate, and tetramethylthiuram disulfide were more inhibitory at 10^{-3} *M* for both amylases and for catalase than for polyphenol oxidase. At a concentration of 10^{-4} *M* these compounds were slightly more inhibitory for catalase than for amylases. However, a tenfold increase in chemical concentration resulted in a proportionally greater increase in inhibition of pancreatic and malt amylases than of catalase.

True solutions containing 10^{-3} *M* of ferric dimethyldithiocarbamate, zinc dimethyldithiocarbamate, or tetramethylthiuram disulfide could not

TABLE II
COMPARATIVE INHIBITION OF AMYLASES, CATALASE, AND POLYPHENOL
OXIDASE CAUSED BY VARIOUS FUNGICIDAL CHEMICALS

Group	Chemical	Inhibition, per cent at <i>M</i> concn. of fungicidal chemicals						
		Pancreatic amylase		Malt amylase		Catalase		Poly-phenol oxidase
		10^{-3} <i>M</i>	10^{-4} <i>M</i>	10^{-3} <i>M</i>	10^{-4} <i>M</i>	10^{-3} <i>M</i>	10^{-4} <i>M</i>	10^{-3} <i>M</i>
I	Elemental sulfur	0	5	0	0	1	15	13
II	Sodium diethyldithiocarbamate	17	8	23	2	6	0	79
	Ferric dimethyldithiocarbamate	82	11	100	3	65	14	19
	Zinc dimethyldithiocarbamate	93	33	88	18	51	45	41
	Tetramethylthiuram disulfide	78	15	100	12	43	30	24
III	Phenol	3 (+)	0	7 (+)	5 (+)	17	0	25
	Hydroquinone	87	51	50	10	80	40	20
	8-Hydroxyquinoline	4	9	4	5 (+)	12	4	33
	5,7-Dichloro-8-hydroxyquinoline	59	39	37	2 (+)	76	26	69
IV	<i>p</i> -Benzoquinone	93	93	99	95	51	12	23
	Tetrachloro- <i>p</i> -benzoquinone	100	98	100	100	100	62	4 (+)
	1,4-Naphthoquinone	79	24	100	93	76	48	18
	2,3-Dichloro-1,4-naphthoquinone	93	55	84	68	77	65	10 (+)
V	Zinc sulfate	47	40	5	13	0	0	17 (+)
	Copper chloride	74	48	69	55	0	0	12 (+)
	Ferric chloride	27	2 (+)	51	8	19	1	8
	Potassium chromate	3	0	0	0	0	0	30 (+)
	Copper-zinc-chromate complex	69	48	68	52	0	0	32 (+)
	Mercuric chloride	96	100	100	97	100	92	45
	Phenylmercuric acetate	98	97	97	97	26	29	45

(+) Indicates an increase in enzyme activity over that of controls.

be prepared because of their limited solubilities in water. This undoubtedly accounts in part for the unexpectedly small increases in inhibition of catalase when the concentration of the chemicals was increased. Solubility factors apparently also complicated interaction of these compounds with polyphenol oxidase since each compound reduced polyphenol oxidase activity in an order which appears to be more closely related to their solubility in water than to their chemical reactivity. Metals contained in zinc dimethyldithiocarbamate and ferric dimethyldithiocarbamate did not seem to interfere seriously in interactions which presumably occur between the organic radical of these compounds and the metals of catalase or polyphenol oxidase.

Phenolic compounds, Group III, differed in their interaction patterns with respect to the different enzymes, depending on ring constitution and substituents. Phenol did not inhibit amylases but usually induced small increases in activity over the controls. At 10^{-3} *M* it reduced activity of both polyphenol oxidase and catalase slightly, but on the basis of other tests inhibition was attributed to competitive action in case of polyphenol oxidase and to complex formation with iron in the Protohematin IX prosthetic group in case of catalase.

Substitution of a second hydroxyl group in the 4-position on the ring to form hydroquinone resulted in strong inhibition of both amylases and increased inhibition of catalase. Inhibition of polyphenol oxidase remained about equivalent to inhibition by phenol.

8-Hydroxyquinoline at 10^{-3} *M* reduced the activities of polyphenol oxidase and catalase but had little effect on those of amylases. Chlorination at the 5- and 7-positions to form 5,7-dichloro-8-hydroxyquinoline increased inhibition of all enzymes. Compared to hydroquinone it was much more inhibitory for polyphenol oxidase but less inhibitory for catalase and amylases. Like hydroquinone, it was more inhibitory for pancreatic amylase than for malt amylase.

The quinones, Group IV, were highly inhibitory for both amylases and for catalase, but relatively much less inhibitory for polyphenol oxidase. The chlorinated derivatives increased oxygen uptake as much as 30 per cent in the initial 5 or 10 minutes of polyphenol oxidase activity on pyrocatechol but decreased uptake toward the end of the 30-minute period so that the over-all increase was small. *p*-Benzoquinone and 1,4-naphthoquinone reduced polyphenol oxidase activity to some extent.

Chlorination of *p*-benzoquinone and 1,4-naphthoquinone increased inhibition of pancreatic amylase and of catalase. *p*-Benzoquinone, at 10^{-3} *M*, inhibited catalase 51 per cent while one-tenth of this amount, or 10^{-4} *M*, of tetrachloro-*p*-benzoquinone inhibited this enzyme 62 per cent. 1,4-Naphthoquinone at 10^{-3} *M* reduced catalase activity 48 per cent while 2,3-dichloro-1,4-naphthoquinone caused a 65 per cent reduction.

The relative potency of benzoquinones as inhibitors of the amylases could not be distinguished at a chemical concentration of 10^{-4} *M* since inhibition was essentially complete. Therefore, a series of dilutions was tested and the concentrations of the quinones required to bring about threshold inhibition of each amylase were compared, Figures 2 and 3. Tetrachloro-*p*-benzoquinone at concentrations of 5×10^{-9} and 5×10^{-8} *M* brought about threshold inhibition of malt and pancreatic amylases, respectively. Concentrations of *p*-benzoquinone, 1,4-naphthoquinone, and 2,3-dichloro-1,4-naphthoquinone required to produce threshold inhibition of malt amylase were essentially equivalent, ca. 5×10^{-7} *M*. However, about 5×10^{-6} *M*, or 10 times as much 1,4-naphthoquinone as 2,3-dichloro-1,4-naphthoquinone was required to produce threshold inhibition of pancreatic amylase. Chlorination of *p*-benzoquinone appeared to have a relatively greater effect on inhibition of malt amylase than on that of pancreatic amylase. Chlorination of 1,4-naphthoquinone, on the other hand, appeared to produce a relatively greater effect on pancreatic amylase inhibition than on malt amylase inhibition. Breaks in the normal sigmoid character of inhibition curves obtained for 2,3-dichloro-1,4-naphthoquinone occurred at about 10^{-5} *M* and were found to be correlated with the solubility of this compound in water; hence, only the lower portion of the curves was considered in comparisons with other compounds. Tetrachloro-*p*-benzoquinone was also very slightly soluble in water but since it inhibited in small quantities, normal sigmoid curves were obtained.

Metal-containing compounds, Group V, differed widely in interaction patterns. Specific metals likewise exhibited large differences with respect to comparative inhibitory activities against the same enzyme. Zinc sulfate, copper chloride, copper-zinc-chromate complex, and potassium chromate increased activity of polyphenol oxidase, while mercuric chloride and phenylmercuric acetate reduced activity, both to an equal extent. Ferric chloride was also slightly inhibitory.

Copper chloride, copper-zinc-chromate complex, potassium chromate, and zinc sulfate had no effect on catalase activity. Ferric chloride and phenylmercuric acetate inhibited slightly, while mercuric chloride produced strong inhibition, even at a concentration of 10^{-4} *M*.

With the exception of potassium chromate, all metal-containing compounds inhibited both amylases. Zinc sulfate was more inhibitory for pancreatic amylase than for malt amylase. Both copper-containing compounds inhibited the amylases to about equal extents, while ferric chloride inhibited malt amylase to a slightly greater extent than it inhibited pancreatic amylase.

Both mercury-containing compounds were particularly inhibitory for pancreatic and malt amylases and their potency could not be distinguished at 10^{-4} *M*. Other tests were carried out with serial dilutions in order to

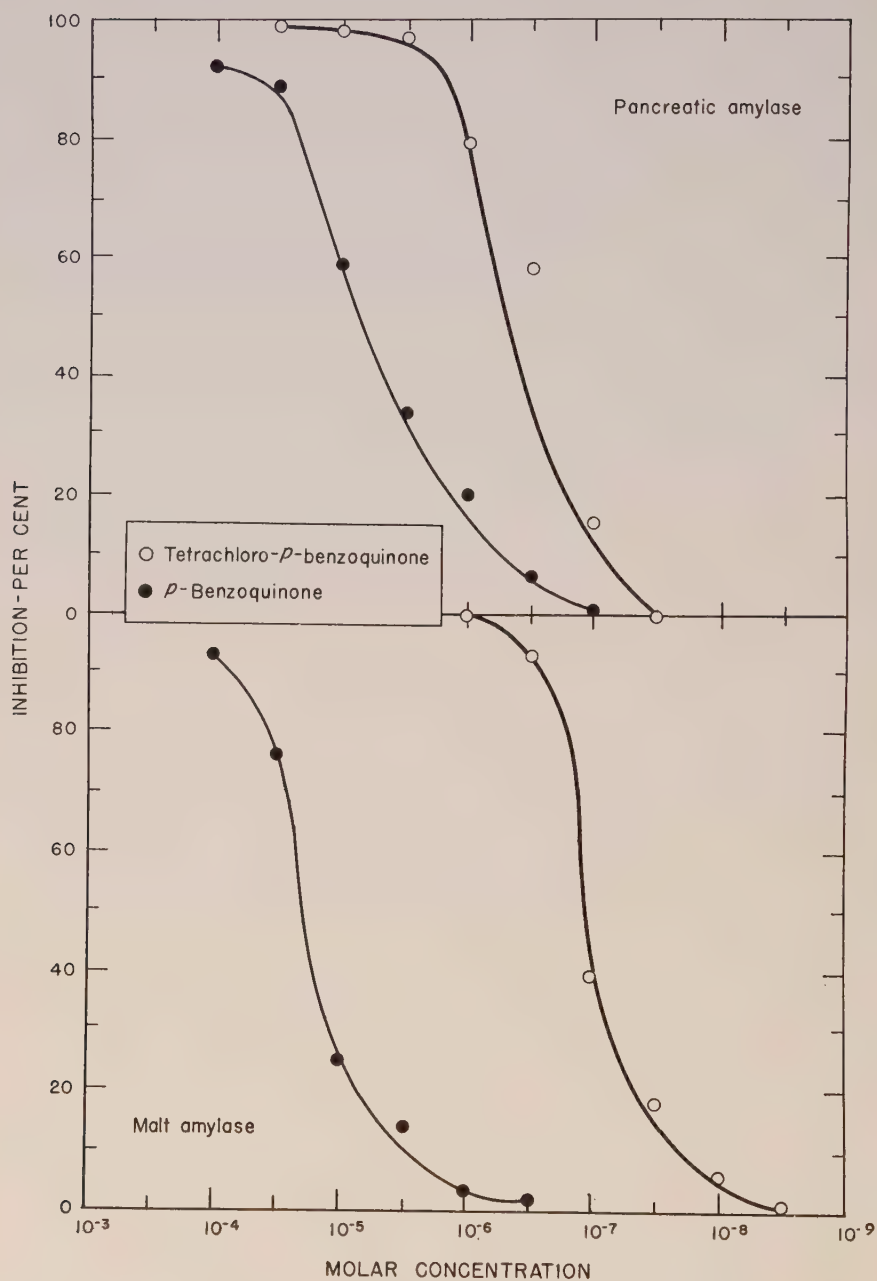


FIG. 2. The effect of chemical concentration on inhibition of pancreatic and malt amylases by quinones.

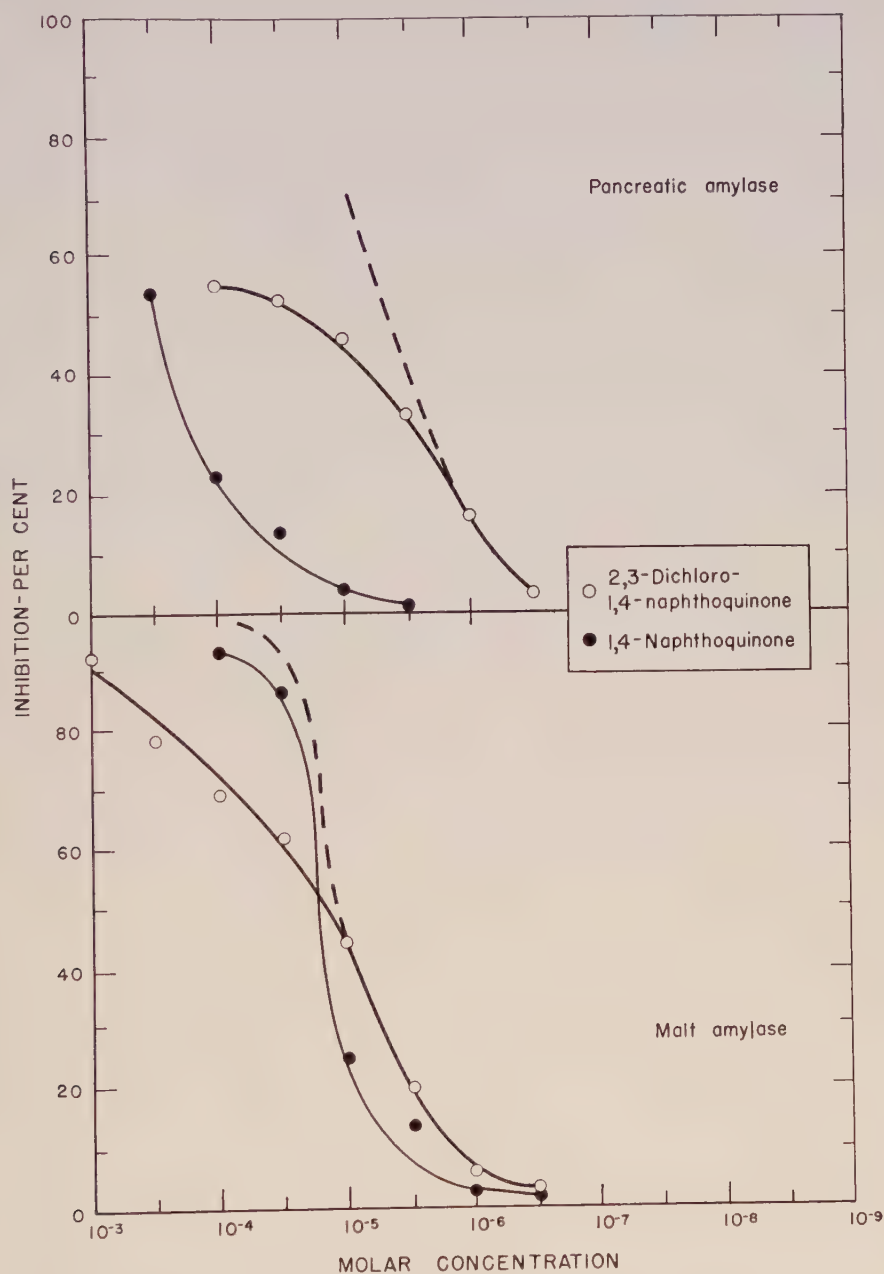


FIG. 3. The effect of chemical concentration on the inhibition of pancreatic and malt amylases by naphthoquinones.

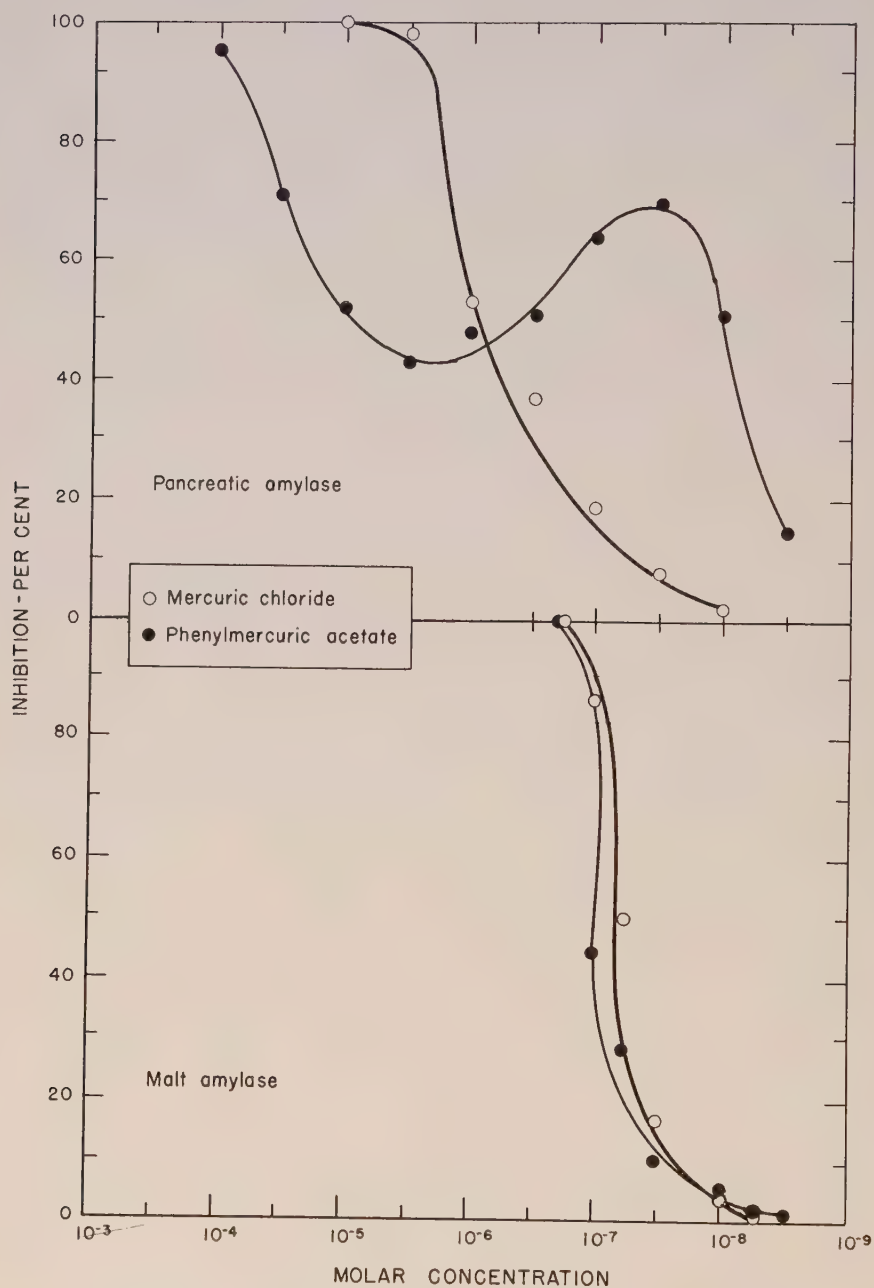


FIG. 4. The effect of chemical concentration on inhibition of pancreatic and malt amylases by mercuric chloride and phenylmercuric acetate.

determine the concentrations required to bring about threshold inhibition of each amylase, Figure 4. Mercuric chloride and phenylmercuric acetate were about equally inhibitory for malt amylase and the characteristics of inhibition were almost identical. The characteristics of their inhibition of pancreatic amylase, on the other hand, were markedly dissimilar. Phenylmercuric acetate produced an inhibition curve with two maxima while mercuric chloride produced the typical sigmoid inhibition curve. Moreover, the concentration of phenylmercuric acetate required to bring about threshold inhibition was about one-tenth that of mercuric chloride required for threshold inhibition (about 7.5×10^{-10} compared to 7.5×10^{-9} M). However, more than 10 times as much phenylmercuric acetate as mercuric chloride was required to bring about complete inhibition. While the concentration of mercuric chloride and phenylmercuric acetate required for threshold inhibition of the two amylases differed only tenfold or less, the concentrations of the compounds required to inhibit pancreatic amylase completely were, respectively, of the order of 100 and 1000 times that required for complete inhibition of malt amylase.

REVERSAL OF INHIBITION

Quinones have not been reported previously to inhibit pancreatic amylase strongly. Hoffmann-Ostenhof and Biach (14) reported no inhibition by several quinones and a low order of inhibition by 2,6-dichloro-*p*-benzoquinone, isonaphthazarin, and lawsone. By utilizing an incubation period, however, it has been shown in this paper that 1,4-naphthoquinone and *p*-benzoquinone as well as chlorinated derivatives of these are strongly inhibitory. Since pancreatic amylase is regarded as being dependent upon free amino groups for catalytic activity, tests were carried out to determine whether or not glutathione, cysteine, or alanine were capable of reversing inhibition by quinones at the amino site. Results from these tests are summarized in Table III.

It was found that glutathione and cysteine were highly effective in

TABLE III
EFFECTS OF THIOL AND AMINO ADDITIVES ON INHIBITION OF PANCREATIC
AMYLASE BY QUINONES

Quinone (10^{-4} M)	Inhibition, per cent, with additive at 10^{-4} M			
	None	Glutathione	Cysteine	Alanine
<i>p</i> -Benzoquinone	77	5	10	77
1,4-Naphthoquinone	16	0	1 (+)	16
Tetrachloro- <i>p</i> -benzoquinone	93	1 (+)	7	93
2,3-Dichloro-1,4-naphthoquinone	41	2 (+)	1	41
None	—	4 (+)	2 (+)	0

(+) Indicates increase in enzyme activity over the controls.

reversing inhibition whereas alanine was completely ineffective at the concentration tested. Glutathione reduced inhibition by *p*-benzoquinone from 77 per cent to 5 per cent and completely reversed inhibition by all other quinones. Cysteine was slightly less effective.

Similar attempts to reverse the inhibitory action of sodium diethyldithiocarbamate on polyphenol oxidase were made by additions of metal ions, Table IV. The ions varied in their capacities to reverse inhibition but none reversed it completely. The order of reversal effects was $\text{Ni}^{++} > \text{Co}^{++} > \text{Cu}^{++} > \text{Fe}^{+++}$. This order probably has no significance except to indicate the order of affinity of the ions for the diethyldithiocarbamate radical.

TABLE IV
EFFECTS OF SEVERAL METAL ADDITIVES ON INHIBITION OF POLYPHENOL
OXIDASE BY SODIUM DIETHYLDITHIOCARBAMATE

Additive (10^{-3} M)	Inhibition, per cent	
	Na diethyldithiocarbamate (10^{-3} M)	Control
None	85	
Copper chloride	67	12 (+)
Cobalt chloride	34	10 (+)
Nickel chloride	22	4
Ferric chloride	76	8

(+) Indicates increase in enzyme activity over the controls.

The fact that none of the ions completely reversed inhibition and that all dithiocarbamate acid derivatives inhibited amylases indicated that some property other than metal complex formation with the copper of polyphenol oxidase or with the iron of catalase contributed to their inhibitory action. A test was carried out, therefore, to determine the capacity of these compounds to act as reducing agents. The results from this test, in which the reduction of alkaline potassium ferricyanide was determined for each of the derivatives, are shown in Table V. All derivatives reduced potassium ferricyanide, but sodium diethyldithiocarbamate was much less active than the other members of the group. This is in accord with the inhibition of amylases.

TABLE V
REDUCTION OF POTASSIUM FERRICYANIDE BY 10 ML. OF 10^{-3} M
DITHIOCARBAMIC ACID DERIVATIVES

Compound	0.05 N $\text{K}_3\text{Fe}(\text{CN})_6$ reduced, ml.
Tetramethylthiuram disulfide	16.0
Sodium diethyldithiocarbamate	3.8
Zinc dimethyldithiocarbamate	16.0
Ferric dimethyldithiocarbamate	14.4
None	0

Tests on the effects of chlorinated quinones on polyphenol oxidase suggested that the oxidase activity was stimulated by these compounds or that they might be acted upon by the oxidase. Experiments were conducted to test these, other quinones, phenol, and hydroquinones as substrates for polyphenol oxidase. Results showed that none of the quinones were oxidized but that phenol was oxidized at a rate about 60 per cent of that of pyrocatechol. Hydroquinone was oxidized to a negligible extent. These tests suggest that phenol, hydroquinone, *p*-benzoquinone, and 1,4-naphthoquinone inhibit polyphenol oxidase by competing with pyrocatechol for the active enzyme site.

DISCUSSION OF RESULTS

Inhibition of enzymes by chemical agents may be the result of any one or a combination of interactions involving active enzyme groups, protein precipitation, masking of active groups, or competition with substrate for active enzyme sites. Compounds used in tests reported in this paper bear little resemblance to the substrates of amylase or catalase and are assumed to interact with the active groups of these enzymes. The quinones, phenol, and hydroquinone, on the other hand, are very similar in many respects to pyrocatechol or oxidation intermediates and could compete for the catalytic surface of polyphenol oxidase. Interactions of this type, while extremely important in chemotherapeutic mechanisms, would not appear to be the most important types of interactions in which quinones can participate in living systems since the quinones are more effective in reducing the activities of enzymes of the amylase types.

Inhibition of polyphenol oxidase by dithiocarbamic acid derivatives would appear to be an example of dual inhibitory processes; namely, complex formation with the copper atoms of the enzyme molecule and a strong tendency on the part of the dithiocarbamic acid derivatives to donate electrons to that center. Other workers have shown that sodium diethyldithiocarbamate forms stable complexes with the copper atoms of all copper-containing enzymes (10, 22, 36). However, since only partial reversal of inhibition of polyphenol oxidase could be brought about in the present tests by addition of metal ions, including copper, copper complex formation must account only partially for inhibition. Other inhibitory properties are indicated by inhibition of amylases reported here and of flavin enzymes (33) reported by others. The nature of these properties is suggested by the capacity of all derivatives tested to reduce potassium ferricyanide and to react with glutathione, as shown by Weed.³ The reaction of these compounds with oxidizing agents, reducing agents, and complex formation with metals suggests some possible mechanisms by which dithiocarbamic acid derivatives may inhibit various enzymes, both *in vitro* and *in vivo*. The catalytic activity or contact of dithiocarbamate-

³ Weed, Richard M. Personal communication.

sensitive enzymes with their substrates would seem to depend on free electron shifts between sulphydryl and amino groups and substrate molecules or between the oxidized and reduced states in case of flavin- or metal-containing oxidases. Donation or acceptance of electrons to or from these centers by dithiocarbamic acid derivatives or metal complex formation could effectively prevent the necessary electron shifts in every case.

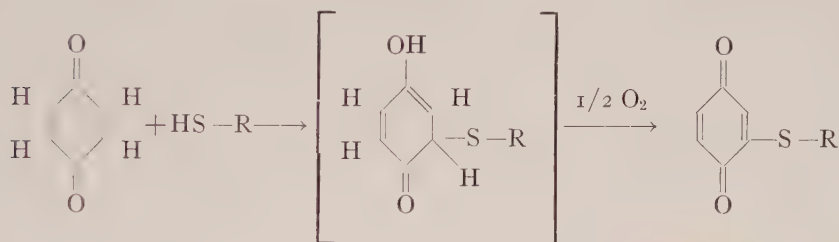
Phenolic compounds differ widely in their inhibitory properties depending upon the nature of the ring and substituents on the ring. Inhibition of amylases and catalase appears to involve the hydroxyl group and/or the ortho position whereas inhibition of polyphenol oxidase appears to involve the whole ring as well as the hydroxyl group since unsubstituted quinones, hydroquinone, and phenol inhibit this enzyme to about an equal extent. Inhibition of catalase and amylases, on the other hand, is increased by formation of hydroquinone which may undergo oxidation through loss of one electron to form semiquinone. Sumner (42, p. 883) attributes the inhibitory activity of hydroquinone against urease to benzoquinone formation. The inhibition percentage obtained for amylase would indicate the presence of a greater concentration of benzoquinone than can be attributed to contaminants. This suggests that inhibition may involve a two step process at the enzyme surface, the first a tautomerization or removal of hydrogen from a hydroxyl group and then interaction as the semiquinone. The first step would probably be the rate determining one and would account for the relatively greater inhibitory action of quinones.

The effects of chlorination on the potency of quinones and 8-hydroxyquinolines as amylase and catalase inhibitors indicate an intensification of reaction tendencies inherent in the hydroxyl or carbonyl groups and the ortho position. Inhibition by 5,7-dichloro-8-hydroxyquinoline is similar to that of hydroquinone with respect to amylases and catalase, which suggests that similar changes may occur in the molecule as it comes in contact with the enzyme. Chlorination appears to facilitate these changes, since 8-hydroxyquinoline shows relatively little inhibitory action.

While the action of 5,7-dichloro-8-hydroxyquinoline on amylases reflects certain properties of this compound similar in nature to those of hydroquinone the action of both 8-hydroxy compounds on polyphenol oxidase indicates a property not possessed by hydroquinone; namely, strong interaction with the copper atom of polyphenol oxidase, in which a chelate complex is formed (22). Inhibition of both polyphenol oxidase and catalase suggests that chlorination increases the chelate strength of the complex. Complexes with iron are known to form with all phenolic and quinone compounds tested and on the basis of catalase inhibition the complexes appear to be strengthened by chlorination. The mechanism of complex formation, while not clearly understood, appears to involve the hydroxyl or carbonyl group which forms coordinate bonds with trivalent iron of the heme group of catalase (47). Data now on hand show that hy-

droxy compounds and quinones participate in at least three different types of interactions with enzymes, all of which are potentially important in living systems; namely, interaction with sulfhydryl groups, interaction with amino groups, and interaction with certain metal atoms of enzymes.

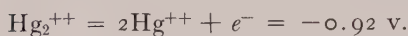
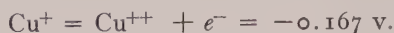
Both amylases are highly sensitive to quinones. Although the details of quinone attack on sulfhydryl and amino groups remain to be elucidated, quinone participation in reactions with thiol groups is generally regarded as an example of the general reaction between thiols and $\alpha:\beta$ -unsaturated ketones, and probably proceeds thus (21):



Interaction between quinones and amino groups probably proceeds in the manner presented for transamination reactions by Braunstein and Kritzman (8) involving Schiff's base or by the mechanism proposed by Karrer and Brandenberger (16) involving reduction to an imino acid with subsequent oxidation. Schönberg *et al.* (38) also found that reaction of certain aldehydic and ketonic compounds with amino acids results in decarboxylation and degradation of the amino acids to the corresponding aldehydes and ketones with one less carbon atom. It is likely that inhibition of the enzyme occurs in the initial transition stages of these reactions and, therefore, does not reflect the extent to which the complete reaction proceeds.

Enzymes of the amylase types are much more sensitive to metals than the copper- and iron-containing enzymes, which shows that active sulfhydryl and amino groups are readily attacked by metal ions. Reaction of metals at these centers in case of polyphenol oxidase or catalase does not affect activity except in case of mercury which probably causes precipitation and denaturation of the protein moiety.

Copper, mercury, and ferric ions attack the sulfhydryl group of malt amylase more readily than do zinc ions. Since the valence of Zn^{++} , Cu^{++} , and Hg^{++} are equal, each of these ions must possess other properties which are inherently different. Zn^{++} exists only as the divalent ion, whereas Cu^{++} , Fe^{+++} , and Hg^{++} have the ability to shift in valence with the acquisition of one electron. These valence shifts furthermore are accompanied by changes in electrical potential characteristically different for each ion (18):



While inhibition is not necessarily correlated with the magnitude of the voltage change, as shown in the case of iron, but must involve other factors, it may help to account for the differences in the behavior of metal ions toward proteins, especially those containing sulfhydryl groups. For further discussion of the relation of oxidation potential to sulfhydryl interaction the reader is referred to the review by Barron (6).

Inhibition of the amylases by mercuric chloride and phenylmercuric acetate presents some interesting contrasts. Both compounds inhibit malt amylase with essentially the same characteristics, which suggests that mercury in both forms interacts with the enzyme in essentially the same manner and that the phenyl radical has little or no influence on the interaction. Inhibition of pancreatic amylase, on the other hand, suggests that mercuric chloride and phenylmercuric acetate react with phosphates. The slope of the inhibition curve for mercuric chloride and pancreatic amylase is markedly less steep than that for malt amylase, probably indicating that the ionic freedom of Hg^{++} is relatively more reduced in phosphates than in acetates as the mercury concentration is increased.

Inhibition of pancreatic amylase by phenylmercuric acetate assumes a complex profile very different from that by mercuric chloride. Although not enough data are on hand at present to explain this curve, it was noted that at a concentration of about $5 \times 10^{-6} M$ of phenylmercuric acetate an insoluble precipitate appears in phosphate buffer, which indicates a reaction between phosphates and phenylmercuric acetate, probably with formation principally of phenylmercuric phosphate. This suggests that at concentrations above $5 \times 10^{-6} M$ inhibition is a function of the solubility of the phenylmercuric phosphate that forms. At concentrations between $5 \times 10^{-6} M$ and $5 \times 10^{-8} M$ no visible precipitation of phenylmercuric phosphate occurs which results in a condition resembling supersaturated solutions. In this case, however, the condition is probably a result of high dilution and failure of the phenylmercuric ion to react with phosphates as dilution becomes greater. At $5 \times 10^{-8} M$ the active form of phenylmercuric acetate or phosphate reaches a maximum as does inhibition. At greater dilution inhibition decreases in proportion to the concentration of phenylmercuric acetate added.

OBSERVATIONS ON IN VITRO SENSITIVITY OF ENZYMES AND IN VIVO TOXICITY WITH REGARD TO FUNGICIDES

The data reported here show that fungicides and compounds closely related to fungicides have a number of properties contributing to enzyme inactivation and that these properties are very difficult to integrate and to

analyze. However, judicious selection of chemical types and analysis of the sorts made here can provide valuable insight into interaction patterns. These interaction patterns, while subject to various modifying influences in living cells, such as penetration rates, detoxification mechanisms, and stability phenomena, can undoubtedly indicate the types of *in vivo* interactions operative in toxicity expressions, if viewed from the standpoint of the sensitivity of various enzyme types. The validity of this concept, of course, depends on how nearly *in vitro* interactions approximate those *in vivo*. It seems reasonable to assume that as the concentration of a toxicant increases within the fungus spore or cell, the most sensitive enzyme will be the first to exhibit inhibition effects. If the enzyme is vital to a metabolic process, the whole process will thus be impeded. It is conceivable that at the threshold concentration of a chemical agent producing growth inhibition only one enzyme may be seriously inhibited if the sensitivity differential between enzymes is great. On the other hand, it seems more likely that several enzymes with a small sensitivity differential may be affected simultaneously by compounds of the types dealt with here.

For instance, it would be exceedingly difficult on the basis of threshold growth inhibition by mercuric chloride to distinguish between inhibition of enzymes with malt or pancreatic amylase characteristics whereas it should be relatively easy to distinguish between these types of interactions and that which takes place between mercuric chloride and polyphenol oxidase and catalase.

While the sensitivity of enzymes of the amylase types to mercurials and quinones *in vitro* is great enough to account satisfactorily for the *in vivo* action of these compounds, the concentrations of dithiocarbamates and hydroxy compounds required to inhibit polyphenol oxidase and catalase leave some doubt as to the role of these particular types of enzymes in *in vivo* interactions. However, if the relative enzyme concentrations in *in vivo* and *in vitro* systems and accumulation phenomenon exhibited by fungus spores (24, 25, 28) are considered, it seems not unlikely that these types of interactions might explain observed toxicity phenomena.

Additional work on other enzyme types and on these and other chemical types is in progress.

SUMMARY

The effects of dithiocarbamates, quinones, phenols, and metal compounds on activity of amino-, sulfhydryl-, copper-, and iron-dependent enzymes (pancreatic amylase, malt amylase, polyphenol oxidase, and catalase, respectively) were found to vary with the properties of the chemicals and with the nature of the active enzyme group. Sulfur was relatively ineffective as an inhibitor of any of the enzymes tested. Dithiocarbamic acid derivatives inhibited all enzymes tested and evidence was presented or cited to indicate that inhibition resulted from complex forma-

tion with metals of metal-containing enzymes and by interference in electron shifts. Inhibition by phenolic compounds depended upon ring constitution and substitutions. Chlorination or substituents which make quinone formation possible enhanced inhibition of all enzymes tested except polyphenol oxidase. Inhibition of pancreatic amylase by phenylmercuric acetate as a function of chemical concentration reached two maxima whereas inhibition by mercuric chloride varied in the normal manner with chemical concentration. Inhibition of malt amylase by both phenylmercuric acetate and mercuric chloride varied with chemical concentration in the normal manner. Quinones were potent inhibitors for both amylases and moderately inhibitory for catalase. Inhibition of amylases was markedly increased by incubation of the enzymes with quinones whereas relatively much smaller increases resulted from incubation of the enzymes with mercury or copper ions.

Pancreatic amylase inhibition by quinones was completely reversed by glutathione or cysteine. Polyphenol oxidase inhibition by sodium diethyldithiocarbamate was only partially reversed by the metal ions tested.

Inhibition is discussed with regard to its implications about the properties of the chemicals involved and interaction mechanisms. Possible relationships between these properties and mechanisms and *in vivo* toxicity are pointed out.

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A SIMPLE, RAPID BIOLOGICAL METHOD FOR DETERMINING THE RELATIVE VOLATILITY OF ESTERS OF 2,4-D AND 2,4,5-T

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INTRODUCTION

The greater hazards associated with the manufacture and practical use of esters of 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), as compared with their salts, have been discussed in several reports (1, 2, 4, 5, 6, 7, 8, 11, 12). It is generally agreed that the relative hazard of herbicides such as 2,4-D depends largely on the effect of their vapors on plants. If the vapors induce adverse effects, the herbicide is considered hazardous. The isopropyl and butyl esters of 2,4-D are examples of hazardous herbicides. In contrast, an amine salt of 2,4-D is considered non-hazardous since no adverse effects are produced on plants when they are confined with the amine salts. From a practical standpoint, the relative volatility of herbicides is based on the physiological activity of their vapors and the sensitivity of the plant affected, without having any direct relation to volatility in the physical sense. In the present report "volatility" refers to the physiological activity of vapor given off by a specified amount of a herbicide or growth regulant applied to filter paper, as indicated by the magnitude of certain responses induced on tomato plants. Thus, the lower the dose required to induce a given response, the higher the "physiological volatility" of the herbicide in question. The term "physiological volatility" is believed to be more inclusive than "phytotoxic volatility" (1) or "formative volatility" (2).

In view of the recognized high volatility of the butyl and isopropyl esters of 2,4-D, attempts have been made in recent years to formulate esters of much lower volatility without reducing their herbicidal effectiveness. The question of how much reduction in volatility is required to make one kind of ester much less hazardous than another is a subject of immediate interest.

Results of tests involving the use of sensitive plants, such as the potato (3), tomato (9, 10, 12, 13), bean (6), and cotton (5), indicate that the physiological volatility of different esters can be detected and measured quantitatively in terms of the magnitude of one or more responses induced by the vapors. Responses induced on tomato plants by vapors of esters of 2,4-D and 2,4,5-T (11, 12) were the same as those induced by esters, acids, and amine salts applied as solutions or emulsions to the aerial parts, or to the soil in which the test plants were growing. The simplest and most

effective method of several tried was the one previously mentioned (12) which consisted of enclosing a tomato plant, together with a measured quantity of an ester deposited on a piece of filter paper, in a No. 20 paper bag for a period of 24 hours or less at temperatures of 70° to 80° F.

The present report gives a more detailed account of results previously mentioned (12) including illustrations of responses on treated plants, and the results of more extensive comparative tests with the isooctyl and isopropyl esters of 2,4-D. Dosage-response curves and illustrations of responses on plants treated with the vapors of 2,4-D and 2,4,5-T have not appeared in previous reports.

MATERIALS AND METHODS

Chemicals. Herbicidal formulations were the same as those used previously (12). These include the following esters and amine salts of 2,4-D and 2,4,5-T: butoxyethyl (American Chemical Paint Co.), isooctyl (Pittsburgh Agricultural Chemical Co.), propylene glycol butyl ether esters (Dow Chemical Co.), ethoxyethoxypropyl (Kolker Chemical Works), alkanolamine (Dow Chemical Co.). In addition to these were the butoxypropyl (Dow Chemical Co.), tetrahydrofurfuryl (Pittsburgh Agricultural Chemical Co.), and Pentasol (American Chemical Paint Co.) esters of 2,4-D. Each of the undiluted formulations contained the active chemical equivalent to four pounds of 2,4-D or 2,4,5-T acid per gallon, except the isopropyl ester of 2,4,5-T which contained an amount of this ester dissolved in acetone equivalent to five pounds of 2,4,5-T acid per gallon. Percentage concentrations are expressed as the free acid equivalent on a volume basis.

Test plants. Greenhouse-grown Bonny Best tomato (*Lycopersicon esculentum* Mill.) plants in 4-inch pots were selected for testing when they were 2.5 to 3 inches in height with three moderately developed leaves. The third leaf was about 3 inches in length on plants 25 to 30 days old from seed. Substantially older or larger plants used in certain experiments were less sensitive and less suitable for enclosing in the paper bags. Test plants were grown before and after treatment at greenhouse temperatures of 70° to 85° F. during the day and at minimum temperatures of 68° to 70° F. at night.

Standard application technique. The bag technique (12) used in most tests consisted of enclosing in a No. 20 paper bag one tomato plant, together with a Whatman No. 1 filter paper (7 cm.) on which was deposited a measured quantity of test solution (0.001 to 0.1 ml.) or distilled water in the case of controls. The No. 20 bag had a volume of 10 liters when open and 5.5 liters when closed (Fig. 1A). In order to avoid accidental contamination, strict adherence was made to the following procedures: (a) one plant at a time was labeled and placed to one side in the bottom of a fully



FIGURE 1. (A) Illustration of equipment and method used for exposing a tomato plant to vapors of a solution of ester deposited on filter paper by means of bacteriological inoculating loop shown in right foreground. (B) Responses on plants 13 days after exposure to different amounts of the isopropyl ester of 2,4-D. (C) Responses 9 days after exposure to different amounts of the propylene glycol butyl ether esters of 2,4-D.

opened upright bag; (b) the bag with the plant inside was carried to the laboratory hood; (c) the test solution was deposited near the center of a filter paper by means of a bacteriological inoculating loop of 0.001 or 0.01-ml. capacity, a swirling motion being used to remove all solution from the loop. A volume of 0.1 ml. was applied as ten separate applications of 0.01 ml. to different areas on the filter paper, and 1 ml. was applied with a micropipette to a crumpled piece of filter paper large enough to prevent the run-off of the test solution; (d) the filter paper was grasped by the edge and placed in the free space on the bottom of the bag, care being taken not to touch the pot, the plant, or sides of the bag; (e) the bag was closed by twice folding the top back and securing with paper clips (Fig. 1A); (f) the closed bag was grasped with both hands at the position where the pot was and carried to a laboratory table where it remained for treatment periods of 24 hours or less at room temperature (70° to 80° F.); (g) between each application of different test solution the loop was washed successively with hot water and acetone and then flamed; (h) after a specified period of exposure the bags were returned one at a time to the hood where the bag was opened, the plant removed, and the bag and filter paper destroyed; (i) the treated plant was then carried to a table; and (j) after all plants in a given test had been removed from the bags, the plants were carried to the greenhouse where responses could be observed and evaluated.

Other application techniques. Several different methods of exposing plants to herbicidal formulations were tried before adopting the technique just described. These included the use of plant containers of a different size made of paper, wax-lined paper, Vinylite, or glass. Test solutions placed in holders of a different size and shape made of glass or of aluminum foil were compared with the filter paper method.

The relative activity of esters applied directly to test plants was determined in one of two ways. One method consisted of first diluting the ester formulation to 0.1 per cent (acid equivalent) before applying 0.01 ml. to one leaflet on a tomato plant in the greenhouse. Another method involved applying 0.001 ml. of the undiluted ester formulation to one leaflet on a tomato plant in the laboratory, the plant being held in the laboratory for two hours, after which the treated leaf was cut off and the plant transferred to the greenhouse. Undiluted ester formulations were not carried to or used in the greenhouse. Formulations not supposed to contain 2,4-D, but which were suspected of being contaminated with 2,4-D, were tested by the method of direct application just described.

Evaluation of responses. Responses were evaluated essentially the same as described previously (12). Curvature of stems was measured with a protractor, and increase in height was measured in centimeters. Curvature of leaves, modification, and stem proliferation were each estimated on the basis of a magnitude of response scale ranging from slight (+) to a maxi-

num (++++). Numerically equivalent values suitable for use in constructing dosage-response curves were obtained by considering each + as 1 and multiplying the sum of the response values for two plants by a factor of 5. Thus the numerical equivalents of 5 and 40 represent minimum and maximum responses respectively (Table I). Measurements of proliferation were based on the relative percentage of the stem that was proliferated. A value of 1 was assigned to each 25 per cent of stem length showing proliferation. Again values for two plants were added and the sums multiplied by 5, giving a range of 5 to 40 from minimum to maximum response.

Curvatures on leaves (epinasty) and stems were evaluated at the end of the exposure period or within 24 hours after removing treated plants from the paper bags. Modification, proliferation, and increase in height were generally evaluated 7 to 10 days after treatment. The magnitude of responses evaluated later than 10 days after treatment was different from that evaluated earlier. Dosage levels high enough to inhibit growth generally induced curvatures greater than 40 degrees, and frequently caused a reversal in the direction of bending at various times within 48 hours after treatment. Consequently, low values for curvature were obtained for plants which were measured at the time the stem was passing through or near the vertical position (Table II). The other responses were not subject to this type of variation. Measurements of stem bending were made within 24 hours after treatment so as to record initial curvatures on plants which recovered from near minimum doses in 24 to 48 hours.

RESULTS WITH ISOPROPYL ESTER OF 2,4-D

RESULTS ACCORDING TO METHOD OF TREATMENT

During the course of the investigation several methods were used for exposing tomato plants to vapors of esters or other growth regulants. The method of treatment finally adopted, induced noticeable stem and leaf curvatures and leaf modification after exposure of the test plants for 24 hours or less to a 0.01 to 0.1-ml. deposit on filter paper of the undiluted isopropyl ester formulation of 2,4-D. Approximately the same results were obtained with the No. 20 paper bag and a Vinylite bag of equivalent volume. In contrast, no detectable responses resulted from the use of glass vials (25×50 mm.) as a holder for 0.01 ml. of the test solution. Reducing the height of the vial from 50 to 3 mm. resulted only in slight modification which represented a minimum response.

When increasing amounts of the isopropyl ester of 2,4-D were placed in glass beakers of 50 to 100-ml. capacity, the increase in magnitude of the responses was not proportional (Table III) as in the case of applying the test solution to filter paper. There was only a slight increase in the magnitude of responses when the duration of treatment with the 10-ml. application in a 50-ml. beaker was extended from 4 to 24 hours. An application

TABLE I
EVALUATION OF DIFFERENT RESPONSES INDUCED ON TOMATO PLANTS BY VAPORS FROM 2,4-D ESTERS OF
RELATIVELY HIGH (ISOPROPYL) AND RELATIVELY LOW (ISOCTYL) VOLATILITY

Concn., %	Amount applied, ml.	Relative vola- tility of ester	Responses in 24 hours				Responses after 7 days				
			Stem bend- ing, de- grees	Leaf epinasty		Numer- ical equiva- lent**	Modification		Proliferation		
				Relative amount*	Plant I 2		Relative amount*	Plant I 2	Numer- ical equiva- lent**	Relative amount*	Plant I 2
I	0.001	High Low	0 0	0 0	0 0	+++ +	+++ 0	25 0	0 0	0 0	0 0
	0.01	High Low	0 0	0 0	0 0	+++ 0	+++ 0	25 0	0 0	0 0	0 0
	0.1	High Low	20 0	+++ +	+++ +	40 0	0 +	0 +	+++ 0	+++ 0	20 0
10	0.001	High Low	0 0	0 0	0 0	+++ 0	+++ 0	30 0	0 0	0 0	0 0
	0.01	High Low	10 0	0 0	+	5 0	+++ 0	+++ 0	35 0	0 0	0 0
	0.1	High Low	33 0	+++ 0	+++ 0	40 0	0 +	0 +	+++ 0	+++ 0	30 0
48	0.001	High Low	40 0	+	+	15 0	+++ 0	+++ 0	35 0	0 0	0 0
	0.01	High Low	43 0	+++ 0	+++ 0	40 0	0 +	0 +	+++ 0	+++ 0	20 0
	0.1	High Low	43 0	+++ 0	+++ 0	30 0	0 +	0 +	+++ 0	+++ 0	40 0

* Increasing magnitude of response ranging from + (minimum) to +++++ (maximum).

** The sum of plus signs for two plants treated alike multiplied by a factor of 5.

TABLE II
RESULTS OF REPLICATED TREATMENTS WITH THE ISOPROPYL ESTER OF
2,4-D IN THE SAME AND IN DIFFERENT TESTS

Date treated	Responses* in 24 hours					Responses* after 7 to 10 days							
	Stem bending, degrees		Relative leaf epinasty			Height increase, cm.		Relative amount					
								Modification			Proliferation		
	No. 1	No. 2	No. 1	No. 2	Total	No. 1	No. 2	No. 1	No. 2	Total	No. 1	No. 2	Total
4-Hour exposure to 0.01 ml.													
Oct. 24	30	20	10	10	20	13	11	15	20	35	0	0	0
Oct. 24	20	40	10	10	20	12	10	15	20	35	0	0	0
Oct. 24	40	20	10	10	20	11	10	20	20	40	0	0	0
Oct. 27	20	20	10	5	15	7	11	5	15	20	5	0	5
Nov. 1	10	20	20	15	35	9	13	15	20	35	10	5	15
Nov. 7	10	15	15	20	35	10	9	15	15	30	0	0	0
Nov. 10	10	20	5	5	10	10	12	15	20	35	0	0	0
Dec. 17	20	20	5	5	10	9	5	15	10	25	5	0	5
Average	20.0	21.3			19.4	10.1	10.1			31.9			3.1
24-Hour exposure to 0.01 ml.													
Oct. 24	65	40	15	15	30	6	3	10	0	10	10	20	30
Oct. 24	30	25	20	20	40	6	5	0	0	0	15	10	25
Oct. 24	30	90	20	20	40	7	2	0	0	0	15	20	35
Oct. 27	40	60	20	15	35	4	4	0	0	0	20	20	40
Nov. 19	60	20	10	15	25	6	6	5	0	5	5	15	20
Dec. 9	40	20	20	20	40	2	4	0	0	0	5	10	15
Dec. 5	20	20	20	20	40	2	2	0	0	0	10	15	25
Dec. 16	45	40	20	20	40	5	5	0	0	0	10	10	20
Average	41.4	39.4			36.3	4.8	3.9			1.9			26.3
4-Hour exposure to 0.1 ml.													
Oct. 24	30	10	15	15	30	3	4	0	0	0	20	15	35
Nov. 7	50	45	20	20	40	5	8	0	15	15	15	5	20
Nov. 10	50	20	15	10	25	8	10	10	20	30	10	0	10
Dec. 17	40	30	20	15	35	8	6	5	5	10	15	10	25
Average	42.5	26.3			32.5	6.0	7.0			13.8			25
24-Hour exposure to 0.1 ml.													
Oct. 24	60	80	20	20	40	2	2	0	0	0	20	20	40
Nov. 20	40	90	20	15	35	2	2	0	0	0	20	20	40
Dec. 9	45	30	20	20	40	2	1	0	0	0	20	20	40
Dec. 5	0	40	15	15	30	1	1	0	0	0	20	20	40
Dec. 16	60	60	15	15	30	3	3	0	0	0	20	20	40
Average	41	60			35.0	2.0	1.8			0.0			40

* See footnotes in Table I for method of evaluation.

TABLE III

COMPILED DATA SHOWING LACK OF CORRELATION BETWEEN AMOUNT OF TEST SOLUTION USED AND MAGNITUDE OF PLANT RESPONSES WHEN THE TEST SOLUTION WAS PLACED IN BEAKERS AS COMPARED TO CORRELATION WHEN FILTER PAPERS WERE USED

Time treated, hours	Amount applied, ml.	Method of applying solution	Kind of bag	Responses* in 24 hours		Responses* after 10 days		
				Stem bending, degrees	Leaf epinasty	Height increase, cm.	Modification	Proliferation
4	0.01	On filter paper	Paper	11	20	10	30	0
	0.10	On filter paper	Paper	31	30	9	25	15
	1.00	On filter paper	Paper	45	30	4	0	40
	0.01	In 50-ml. beaker	Paper	58	10	8	30	0
	0.10	In 50-ml. beaker	Paper	25	40	12	30	10
	1.00	In 50-ml. beaker	Paper	35	30	10	30	5
	10.00	In 100-ml. beaker	Vynlite	0	0	8	20	0
8	10.00	In 100-ml. beaker	Vynlite	0	0	9	20	0
24	10.00	In 100-ml. beaker	Vynlite	48	35	7	10	20
24**	0.01	On filter paper	Paper	43	36	4	2	26

* See footnotes in Table I for method of evaluations.

** Results on last line are the averages for eight tests.

of 0.01 ml. of the test solution on filter paper induced more pronounced responses than 10 ml. of the same solution applied in a beaker of 50 to 100-ml. capacity (Table III). Presumably the greater surface of the solution on filter paper resulted in more rapid volatilization.

The size of the paper bag gave comparable results in the range No. 20 to 0.25 bushel with a corresponding range in volume of 5.5 to 16 liters when the bags were closed at the top as in Figure 1 A. However, more pronounced responses were induced by a given amount of test solution confined with the tomato plant in a No. 12 (3 liter) bag as compared with bags of larger capacity (5.5 to 16 liters). Clean bell jars were satisfactory but extremely difficult to free from traces of ester which were capable of causing a minimum response (modification of leaves). Consequently, the use of bell jars is not recommended.

FACTORS AFFECTING VOLATILITY

When test plants were exposed to vapors of the isopropyl ester of 2,4-D, the magnitude of induced responses varied with the duration of treatment, the concentration and amount of ester used, and the temperature. Increasing the level of one or more of these factors caused an increase in the overall effects on the test plant. A minimum effective dose caused only leaf modification. Slightly higher doses caused temporary or permanent curva-

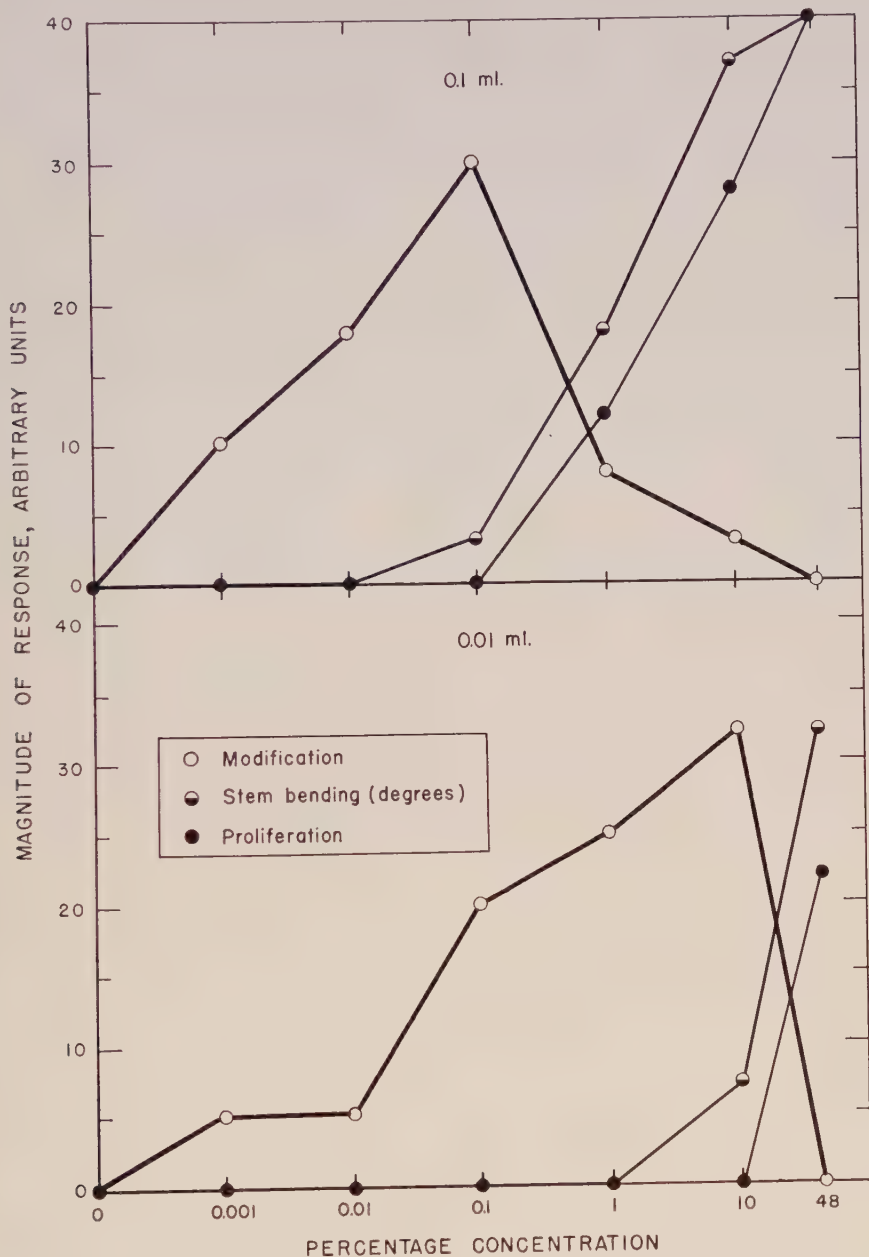


FIGURE 2. Dosage-response curves for three different responses showing the effect of vapors from different concentrations of the solution of isopropyl ester of 2,4-D applied to filter paper. Stem bending was measured at end of 24-hour exposure, and modification and proliferation 7 to 10 days later.

ture of leaves and stem, and a more pronounced degree of modification. The magnitude of modification reached a maximum and then decreased as growth was retarded, until with the higher doses there was no modification due to lack of growth. The appearance of proliferation on tomato stems was associated with a decrease in modification. With increasing doses of 2,4-D the magnitude of proliferation increased throughout the sub-lethal range. Thus the dosage-response curves for modification cross those for stem bending and proliferation as indicated in Figure 2. It is to be noted that the shapes of the dosage-response curves for modification are similar for vapor (Fig. 2) and solution treatment (Fig. 3A).

Quantities of the isopropyl ester of 2,4-D which inhibited terminal growth for at least 7 to 10 days did not prevent some stem elongation. In this case growth was generally resumed shortly after the tenth day so that modified leaves eventually developed on plants which previously had exhibited none due to lack of terminal growth. Thus the evaluation of responses on test plants 7 to 10 days after treatment was not necessarily the same as at a later time. In most cases the results were evaluated 7 to 10 days after treatment started, since little or no difference in the relative magnitudes of modification and proliferation occurred during this 3-day interval.

Concentration and quantity of ester used. The magnitude of responses induced on tomato plants by vapors of the isopropyl ester of 2,4-D depended upon the concentration and volume of solution of ester applied to the filter paper. The influence of each of these factors is shown in Tables I and IV, in Figures 2 and 4, and is illustrated by the responses on test plants in Figures 1 B and 5 A and B. Increasing the volume of test solution from 0.001 to 1 ml. with the resulting increase in the surface exposed, exerted a more pronounced effect on the various responses than increasing the concentration from 0.001 to 48 per cent. Visual evidence of this difference is shown by the responses on test plants in Figure 5 A and B after 6 days. One set of plants photographed after 13 days (Fig. 1 B) shows that the 0.1 and 1-ml. applications of the ester were herbicidal.

Approximate threshold percentage concentrations for responses resulting from a 4-hour exposure of the plants (Table IV) were as follows: 0.001 for modification, 0.01 for stem curvature and leaf epinasty (evaluated 24 hours after treatment started), and 0.1 for proliferation with the 1-ml. application. The corresponding near-threshold values for the volume of solution of ester were: 0.001 ml. of 10 per cent for modification and of 48 per cent for stem curvature, and 0.01 ml. of 10 per cent for epinasty and of 48 per cent for proliferation. Thus modification required the lowest and proliferation the highest dose of the 2,4-D ester. The same relative differences held also for the 24-hour exposure, although the value for each response was higher. A 24-hour exposure to the lowest dose (0.001 ml. of

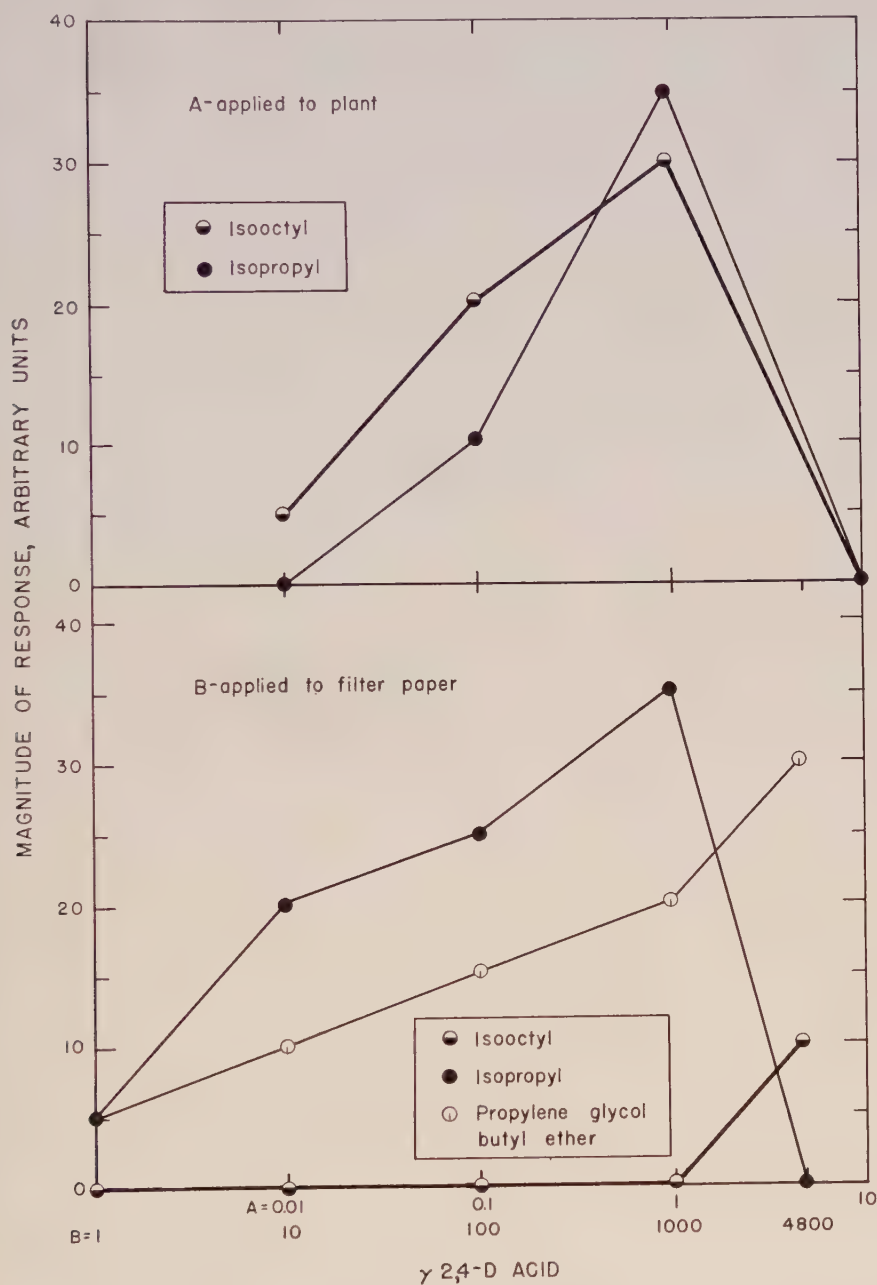


FIGURE 3. Relation between quantity of 2,4-D ester and the magnitude of tomato leaf modification induced in 7 days by 0.01 ml. applications of low (isooctyl) and high (isopropyl) volatile esters when applied (A) as solutions to one leaflet on test plant, and (B) as vapors from ester on filter paper. Abscissa values are γ 2,4-D acid equivalents for different concentrations of the ester, 1 γ for solutions being equal to 1000 γ for vapor.

TALE IV

EFFECT OF CONCENTRATION AND AMOUNT OF ESTER, AND DURATION OF EXPOSURE ON THE RESPONSE* OF PLANTS TO VAPORS OF THE ISOPROPYL ESTER OF 2,4-D

Concn., %	4-Hour exposure					24-Hour exposure				
	Response after 24 hours		Response after 7 days			Response after 24 hours		Response after 7 days		
	Stem bend- ing, degrees	Leaf epi- nasty	Height in- crease, cm.	Modifi- cation	Prolif- eration	Stem bend- ing, degrees	Leaf epi- nasty	Height in- crease, cm.	Modifi- cation	Prolif- eration
1 MI.										
48	45	30	4	0	35	38	25	3	0	40
10	50	20	3	0	30	30	30	4	0	40
1	30	35	5	10	15	68	40	4	0	35
0.1	10	30	5	30	10	60	35	3	5	30
0.01	10	10	6	30	0	20	20	6	30	5
0.001	0	0	4	5	0	0	0	5	20	0
0.1 MI.										
48	35	35	3	0	30	43	30	2	0	40
10	15	30	5	5	15	33	40	4	0	30
1	20	15	6	30	0	20	40	4	0	20
0.1	0	0	5	15	0	5	15	7	30	0
0.01	0	0	4	10	0	0	0	5	20	0
0.001	0	0	5	0	0	0	0	5	15	0
0.01 MI.										
48	20	10	7	25	5	43	40	5	0	20
10	10	10	6	20	0	10	5	6	35	0
1	0	0	5	15	0	0	0	5	25	0
0.1	0	0	5	0	0	0	0	5	20	0
0.01	0	0	5	0	0	0	0	5	5	0
0.001	0	0	4	0	0	0	0	5	10	0
0.001 MI.										
48	10	0	6	30	0	40	15	7	35	0
10	0	0	5	10	0	0	0	6	30	0
1	0	0	5	0	0	0	0	6	25	0
0.1	0	0	5	0	0	0	0	4	10	0
0.01	0	0	5	0	0	0	0	5	10	0
0.001	0	0	5	0	0	0	0	5	15	0

* See footnotes in Table I for method of evaluation.

0.001 per cent) caused modification in this series of tests (Table IV). No attempt was made to determine threshold values on the basis of lower dose ratios. Since the values for stem curvature and leaf epinasty were nearly the same for application of 0.01 to 1 ml., stem bending was used in most cases to represent curvature responses on the basis that it could be measured with greater precision.

Although the shapes of the dosage-response curves for modification are similar for direct treatment of test plants with small amounts of the solution and by vapor treatment, the quantities used on filter paper were 100 to 1000 times those applied on test plants (Fig. 3). For inducing modification 0.1 to 1% 2,4-D was required by direct application and 3 to 1000% by

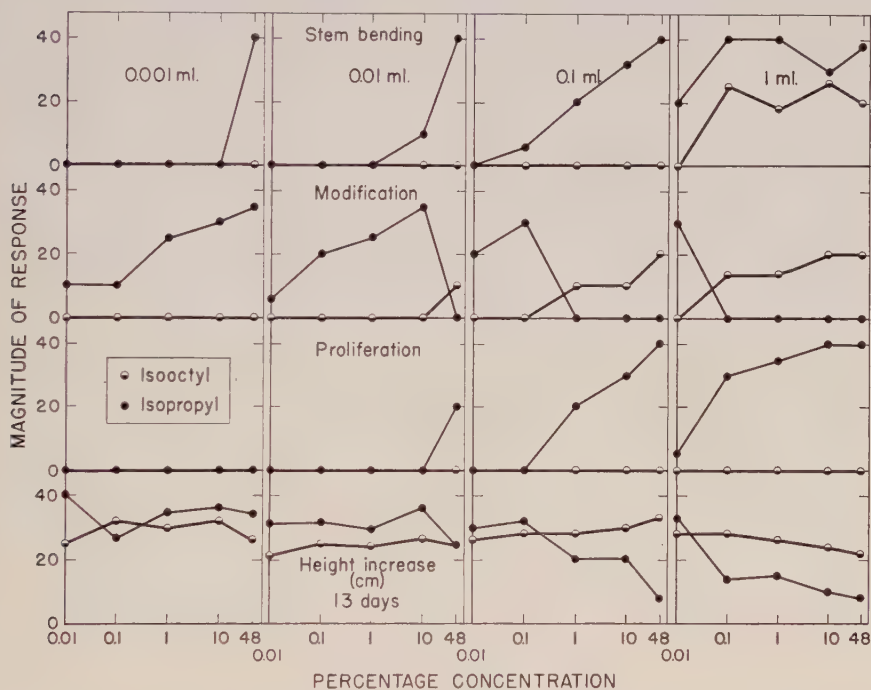


FIGURE 4. Comparative differences in the volatility of the isooctyl and isopropyl esters of 2,4-D during a 24-hour exposure, as represented by dosage-response curves showing the relation between the responses induced on tomato by vapors, and the concentration and volume of the solution of ester applied to filter paper.

the filter paper technique (Fig. 3). This indicates that 0.1 to 3 per cent of the ester vapors from filter paper reached the test plant. In a similar type of test involving treatment of potato tubers, Denny (3) recovered by extraction from the tuber tissue 1 to 5 per cent of the methyl ester of 1-naphthaleneacetic acid added to the filter paper. However, with the potato

tubers the treated filter paper was closer to the test object than in the present tests. The important similarity between these two methods of vapor treatment is that both show a quantitative relation between the quantity of ester applied to filter paper and the magnitude of response on the test object.

Duration of exposure. A given dose of the 2,4-D ester caused increasingly severe effects on test plants with increasing periods of exposure to vapors of the ester. The appearance of the plants 3 days after various periods of exposure to 0.01 ml. of the 48 per cent formulation is shown in Figure 5 C. Plants exposed for 24 hours (not shown) resembled in appearance those exposed for 16 hours. However, this did not apply to the low volatile esters, in which case the plants exposed for 24 hours showed more pronounced responses than those exposed for 16 hours. Results in Tables II and IV also show the greater effectiveness of a 24-hour as compared with a 4-hour exposure.

Results with applications of 0.01 and 0.1 ml. (Table II) showed good agreement for replicate treatments in the same and in different tests, except in the case of stem bending.

Temperature. Although most tests were carried out at room temperature (70° to 80° F.), higher temperatures were used in special tests. Responses were considerably more pronounced on plants treated at temperatures of 110° to 120° F. Responses on plants 3 days after being exposed to two different doses of the 2,4-D ester at temperatures of 75° and 116° F. are shown in Figure 5 D.

COMPARATIVE RESULTS WITH DIFFERENT ESTERS

The alkanolamine salts of 2,4-D and 2,4,5-T gave no response and the following esters of both acids were of relatively low volatility: isooctyl, butoxyethyl, ethoxyethoxypropyl, and propylene glycol butyl ether. In addition, the tetrahydrofurfuryl and butoxypropyl esters of 2,4-D were of low volatility. This is in contrast to the considerably higher volatility of the Pentasol ester of 2,4-D. Vapors from esters of 2,4,5-T induced less pronounced responses than vapors from equivalent amounts of the corresponding 2,4-D esters. These differences are partly explicable on the basis that 2,4,5-T formulations (not contaminated with 2,4-D) did not modify the foliage of tomato plants.

Differences in volatility between the formulations of 2,4-D (Fig. 6 A) and 2,4,5-T (Fig. 6 B) in the low volatile group were determined within 24 hours after treatment, on the basis of initial curvature responses or according to later responses, depending upon the dosage level of the ester and the duration of exposure. Examples of responses on plants 24 hours (Fig. 6), 2 to 6 days (Figs. 5 and 7), and 13 days (Fig. 1 B and C) after initiating treatment show that the magnitude of differences varies with the dosage



FIGURE 5. Responses induced on tomato by vapors of the isopropyl ester of 2,4-D, showing the effect of (A) amount of solution of ester used, (B) concentration with 0.01 ml. application, (C) duration of exposure to 0.01 ml. of 48 per cent, and (D) temperature during 2-hour exposure. Plants in A and B were photographed after 6 days and in C and D after 3 days. Compare with Figure 7.

level of the ester, the duration of exposure, and the time after treatment. Thus it is important to select the proper set of conditions for determining differences in the volatility of different esters. For example, with applications of 0.1 to 1 ml. of the propylene glycol butyl ether and isopropyl esters the responses differed less up to 6 days after treatment (Figs. 5 A and 7 A) than after 13 days (Fig. 1 B and C). Small differences at low dosage levels were dependent upon the magnitude of modification (Figs. 1 B and C and 3 B). Increasing the volume of solution of the ester formulation (Figs. 5 A and 7 A) had a greater effect than increasing the concentration (Figs. 5 B and 7 B).

In all tests to date, the propylene glycol butyl ether ester of 2,4-D was less volatile than the isopropyl ester, and more volatile than the isooctyl ester. When expressed as the quantity of 2,4-D required to induce slight



FIGURE 6. Illustration of method used to distinguish low volatile from high volatile formulations of (A) 2,4-D, and (B) 2,4,5-T, based on curvature responses induced on tomato 24 hours after the start of a 16-hour exposure to the test solution. Left to right in A and B: bag control, 1 ml. alkanolamine salt, 1 ml. butoxyethyl ester, 1 ml. propylene glycol butyl ether ester, 1 ml. isooctyl ester, and 0.001 ml. isopropyl ester.

modification (rating of 10), the propylene glycol butyl ether ester was 480-fold and the isopropyl ester about 2000-fold as volatile as the isooctyl ester (Fig. 3 B), notwithstanding that the isopropyl and isooctyl esters were of equivalent herbicidal activity when applied as solutions directly to the test plant (Fig. 3 A).

In view of the relatively low volatility of the isooctyl ester, comparative tests with this and the more volatile isopropyl ester of 2,4-D, involving a wide range of dosage, were undertaken. Dosage-response curves in Figure 4 show the volumes of 2,4-D ester formulations (equivalent to four pounds

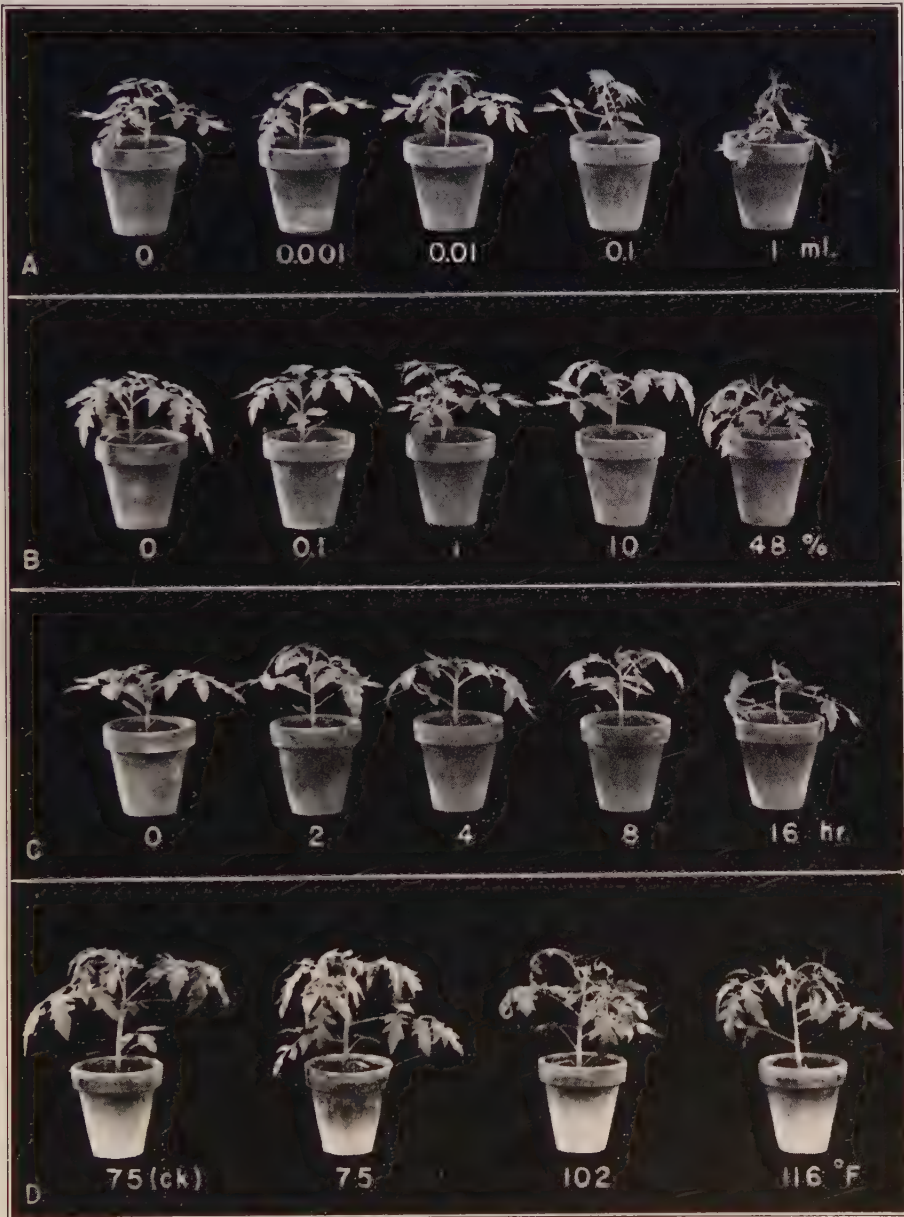


FIGURE 7. Responses induced on tomato by vapors of the propylene glycol butyl ether ester of 2,4-D, showing the effect of (A) amount of solution of ester used, (B) concentration with 0.01 ml. application, (C) duration of exposure to 0.01 ml. of 48 per cent, and (D) temperature during 4-hour exposure. Plants in A and B were photographed after 3 days, in C after 2 days, and in D after 10 days. Compare with Figure 5.

of 2,4-D acid per gallon) that induced maximum or near maximum responses with the isopropyl ester and no responses with the isooctyl ester. These amounts were as follows: 0.001 ml. for modification, 0.001 to 0.1 ml. for stem bending, and 0.1 to 1 ml. for proliferation and inhibition of growth. Similarly, when 1 per cent solutions (equivalent to 0.04 pound of 2,4-D acid per gallon) of the esters were used, 0.1 ml. applications of the isopropyl ester induced noticeable stem bending and proliferation (rating of 20), whereas the same volume of the isooctyl ester induced neither stem bending nor proliferation. Likewise, 0.001 to 0.01 ml. applications of 1 per cent solutions of the isopropyl ester induced noticeable modification (rating of 25), whereas no modification was induced by the same volumes of the isooctyl ester.

In connection with tests designed to detect contamination of herbicidal formulations of low volatility with high volatile esters of 2,4-D, a series of mixtures of isopropyl and isooctyl esters of 2,4-D was prepared. The mixtures contained from 0.001 to 10 per cent isopropyl ester by volume and in each case a total equivalent to four pounds of 2,4-D acid per gallon. The results (Table V) showed that slightly greater than simple additive effects

TABLE V

INITIAL AND FINAL RESPONSES INDUCED ON TOMATO PLANTS BY A 24-HOUR EXPOSURE TO DIFFERENT CONCENTRATIONS OF THE ISOPROPYL ESTER OF 2,4-D IN WATER AND IN MIXTURES WITH THE ISOCTYL ESTER OF 2,4-D

Per cent isopropyl in isooctyl	Reponses* in 24 hours		Responses* after 10 days		Per cent isopropyl in H ₂ O	Responses* in 24 hours		Responses* after 10 days	
	Stem bending, degrees	Relative leaf epinasty	Relative degree			Stem bending, degrees	Relative leaf epinasty	Relative degree	
			Modifi- cation	Prolif- eration				Modifi- cation	Prolif- eration
0.01 Ml.									
0**	0	0	25	0	0†	0	0	0	0
0.001	0	0	5	0	0.001	0	0	0	0
0.01	0	0	10	0	0.01	0	0	5	0
0.1	0	0	20	0	0.1	0	0	20	0
1.0	0	10	30	0	1	0	0	25	0
10	15	30	20	20	10	0	5	30	0
0.10 Ml.									
0**	10	20	30	5	0†	0	0	0	0
0.001	10	5	20	0	0.001	0	0	5	0
0.01	0	0	20	0	0.01	0	0	15	0
0.1	0	10	30	0	0.1	0	0	30	0
1	5	35	0	20	1	8	15	15	5
10	45	40	0	30	10	33	35	5	25

* See footnotes in Table I for method of evaluation.

** 48 per cent isooctyl ester (4 lb. acid per gallon).

† Water controls.

were obtained. Two additional series of tests gave similar results. With the 0.01-ml. application of mixtures containing 10 per cent isopropyl ester, additive effects for stem and leaf curvatures and proliferation ranged from 15 to 30 rating units higher than for the corresponding 10 per cent isopropyl ester or the 48 per cent isooctyl ester control solutions.

The 0.1-ml. applications of mixtures containing 1 to 10 per cent isopropyl ester also gave similar additive effects. The presence of less than 1 per cent of a volatile ester such as isopropyl could not be detected in a non-volatile isooctyl ester. Consequently, less than 1 per cent contamination would not be of practical importance. Mixtures containing 0.001 to 0.01 per cent of the isopropyl ester appeared to be less effective (Table V). The additive and inhibitive effects obtained with vapors from mixtures of these two esters are, in some respects, similar to the results obtained with mixtures of amine salts of 2,4-D and an adjuvant when applied directly to the test plants (4). Mixtures of equal parts of the isopropyl and isooctyl esters with a total concentration equivalent to four pounds of 2,4-D acid per gallon induced the same magnitude of modification as an equivalent concentration of the isopropyl ester used alone.

Results showing the detection of 1 per cent or more of the isopropyl ester of 2,4-D in a mixture with the isooctyl ester (Table V) is of considerable practical importance. In view of the accidental contamination of commercial formulations known to have occurred (11), the present method should prove useful for detecting the possible presence of high volatile esters of 2,4-D in insecticides, fungicides, and low volatile herbicides. Where the high volatile ester of 2,4-D is the only 2,4-D ester in the formulation, as little as 0.01 per cent will modify leaves of tomato (Table V) and cotton (11). Contamination of formulations detected by the filter paper method can be verified by direct treatment of the tomato by means of the droplet method (4), care being taken to observe the precautions described in the present report. The appearance of modified leaves in 7 days would prove the presence of 2,4-D in the formulation. As shown in earlier tests (12), two of the 2,4,5-T formulations were contaminated with 2,4-D.

SUMMARY

A simple, rapid biological method for determining the relative (physiological) volatility of different formulations of 2,4-D and 2,4,5-T is described. The technique consisted of enclosing a tomato plant 2.5 to 3 inches tall, together with a filter paper containing a known amount of test solution, in a No. 20 paper bag for 24 hours or less at 70° to 80° F.

The physiological volatility of an ester was affected by the concentration and amount applied to the filter paper, duration of exposure, and the temperature during treatment. Since the over-all effects on test plants increased with increasing quantities of the ester, relative volatility was de-

terminated in terms of the quantity of ester required to induce a given magnitude of one or more responses. The order of decreasing sensitivity of responses to vapors of esters was leaf modification, curvature of stem and leaves, proliferation of stem and inhibition of growth, and killing. Modification reached a maximum and then decreased as growth was retarded, until with the higher doses there was no modification due to lack of growth.

The shapes of dosage-response curves for the different responses were essentially the same for vapor treatments as for treatment of one leaflet with 0.001 to 0.01 ml. of a 2,4-D formulation. The quantity of 2,4-D ester required to induce a given magnitude of modification was 30 to 1000 times greater for vapor as compared with direct treatment of one tomato leaflet with the 2,4-D ester.

Applications of 0.01 ml. of the formulations (equivalent to four pounds of 2,4-D or 2,4,5-T acid per gallon) and a 24-hour exposure were used to distinguish between esters of low and high volatility. Pronounced curvatures and slight to moderate proliferation were induced by the more volatile isopropyl and Pentasol esters of 2,4-D, as compared with neither curvatures nor proliferation with the low volatile esters (isooctyl, ethoxyethoxypropyl, butoxypropyl, butoxyethyl, tetrahydrofurfuryl, and propylene glycol butyl ether).

Applications of 0.1 and 0.01 ml. were used to distinguish differences between the six low volatile esters of 2,4-D, some inducing slight curvatures and proliferation and others none. The alkanolamine salts of 2,4-D and 2,4,5-T produced no adverse effects when confined with tomato plants at temperatures up to 116° F.

In mixtures of a low (isooctyl) and high (isopropyl) volatile ester of 2,4-D as little as 1 per cent of the high volatile ester was detected. Vapor from 0.01 ml. of 0.01 per cent (17) isopropyl ester of 2,4-D induced slight modification.

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SEED GERMINATION FOR SOME SPECIES OF *PLANTAGO*¹

CLYDE CHANDLER

In the course of an experiment initiated for the improvement of commercially important *Plantago* spp. through induction of polyploidy, hybridization, and selection, it became necessary to investigate the causes of erratic germination of the species under consideration. From published literature on the behavior of seeds of this genus, length of storage period, dormancy, and light sensitivity are factors to be considered. It has been reported that seeds of *P. major*, *P. lanceolata*, or *P. rugelii* will remain viable in dry storage for periods up to 10 years and buried storage in the soil for as long as 40 years [Beal (2), Darlington (4), Duvel (5), Dorph-Petersen (6), Kjaer (7)]. Other reports have dealt with the effect of the age of the seeds of *Plantago* on their dormancy. Kjaer (7) obtained 27 per cent germination of *P. major* seeds in the field and 96 per cent in the laboratory after one month of dry storage. Krug (8) found that seeds of *P. lanceolata* after maturity have a rest period of several weeks, and that seeds of *P. major* show a typical ripening period extending over 14 months. Dormancy of the fresh seeds could be overcome by special germination temperatures, i.e., a daily alternation of 18° to 30° C., or constant temperatures of 24° or 30° C. He concluded that dormancy could be broken by relatively high temperatures. Barton (1), on the other hand, secured the best germination of seeds of *P. fastigiata* at a lower temperature (20° C.). Dry storage of these seeds for nine months after harvest improved their germination markedly. Crocker (3) reported over 95 per cent germination for *P. major* after the seed coats had been broken. High germination temperatures only partly overcame the seed coat effects.

The light sensitivity of *Plantago* seeds has been the subject of study by several workers. Perhaps the most extensive light studies with *Plantago* seeds have been made by Witte (9), who lists 30 different species under three groups: 1. indifferent (same germination in light and dark), 11 species; 2. light germinators (germinate well in light, but very little in dark), 14 species; and 3. dark germinators, 5 species.

All of these reports serve to emphasize the variability of the germination behavior of *Plantago* seeds and the need for determination of germination methods for the species included in the present study, none of which has been reported in published articles. Data presented in this paper show that dormancy in recently-harvested seeds of *Plantago ovata* was overcome by a period of dry storage; germination of *P. rhodosperma* and *P. Wright-*

¹ Supported in part by Johnson-Salisbury, Inc., New York, N. Y.

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iana was favored by light; and germination of seeds of all three of these species was delayed or inhibited by high temperature (30° C.). Seedlings of *P. amplexicaulis* were obtained only by dissection of embryos.

MATERIALS AND METHODS

Seeds of *Plantago ovata* Forsk., collected in 1942, and *P. rhodosperma* Decne. and *P. Wrightiana* Decne., collected in 1948, were received from Johnson-Salisbury, Inc., New York, N. Y. These seeds had been held in dry storage at room temperature. Seeds were germinated on five sheets of moist filter paper in Petri dishes at room temperature. One hundred seeds were used for each test. Seedling progenies were grown from these seeds and new seeds were collected. In the meantime seeds of all three species were treated with colchicine and tetraploids were obtained. Seedling progenies were grown from the tetraploid plants for further seed production. In this paper, data are reported for germination ability of old seeds and freshly-harvested seeds from diploid plants and from tetraploids when sufficient numbers of seed were available.

In addition to room temperature, seeds were tested at constant temperatures of 10°, 15°, 20°, 25°, and 30° C., and greenhouse and daily alternations of 10° to 30°, 15° to 30°, 20° to 30° C., 5° C. to greenhouse, and 10° C. to greenhouse. In all alternating temperatures the cultures were left overnight (16 hours) at the low temperature and 8 hours at the high temperature for 5 days each week. Seeds were tested in both light and darkness. Petri dishes were painted black to insure complete darkness. Two Westinghouse 15-watt fluorescent daylight bulbs were the source of light in the constant temperature rooms. Seed in dark Petri dishes were exposed to light for only a few seconds when germination records were taken.

Seeds of *Plantago amplexicaulis* Cav. from the same source were also tested for germination.

RESULTS AND DISCUSSION

Seeds of *P. ovata*, *P. rhodosperma*, and *P. Wrightiana* collected in 1942 and 1948 gave 61, 93, and 75 per cent germination on moist filter paper at room temperature, when they were tested in 1952. Germination was prompt and usually complete within seven days. Seeds from the same source planted in flats of sphagnum in the greenhouse gave prompt germination and were potted in soil at the end of three weeks. At the time of potting 61, 64, and 42 per cent seedling production was obtained. These percentages were low due to damping-off of the seedlings in the flats. At this time it seemed that the seeds of these three species germinated within seven days after planting and that no special methods were required for germination. However, the germination of seeds from polyploid plants,

sown as soon as they were harvested (September 25), was slow and after 30 days ten of the 40 series planted in soil in the greenhouse gave less than 5 per cent germination. From 12 to 70 per cent germination was obtained for these series after 60 days. Duplicate tests started on October 15, i.e. after the seeds had been in dry storage in the laboratory for 20 days, gave from 43 to 82 per cent germination in one month. It was evident that freshly-harvested seeds needed a period of after-ripening before planting for prompt germination. It seemed advisable before planting other seeds to determine some of the requirements for better germination of seed of these three species.

Plantago ovata

In August 1952, freshly-collected seeds from diploid plants of this species were available and further tests were made to compare the germination of recently-harvested and ten-year-old seeds. Cultures were placed at 20° C., 30° C., daily alternating 20° to 30° C., and room temperature. It may be noted in Figure 1 that old seeds at 20° to 30° C. germinated 99

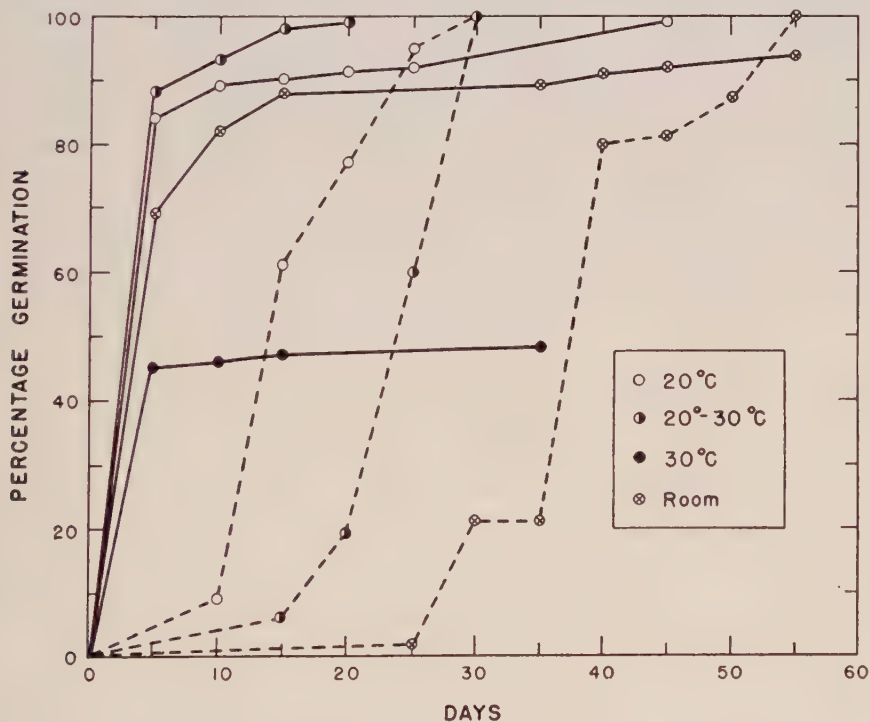


FIGURE 1. Germination of old and freshly-harvested seed of *Plantago ovata* (diploid) at various temperatures. Dotted lines indicate freshly-harvested seed and solid lines indicate old seed.

per cent within 20 days. Germination was very prompt with 82 per cent emergence within three days. In contrast to the earlier test, seeds germinated 94 per cent at room temperature and 99 per cent at 20° C. Good germination was obtained in 20 days at room temperature and 20° C.; however, germination was not complete until over a month later. The higher temperature of 30° C. was less favorable for germination since, after 35 days, only 48 per cent of the seeds germinated.

Seeds collected June–July and tested in August 1952 germinated 100 per cent at 20° C., 20° to 30° C., and room temperature but there was a delay in germination as compared with the old seeds, especially when germination was at room temperature (Fig. 1). None of the fresh seeds germinated at 30° C. These results indicate a dormancy in freshly-harvested seed which is overcome by a period of dry storage.

The effect of light on the germination of old and fresh diploid seeds was tested at constant temperatures of 15°, 20°, 25°, and 30° C., and daily alternations of 10° to 30°, 15° to 30°, and 20° to 30° C. There was no significant difference in the percentage of germination of seeds in light and dark. Germination of fresh seeds was delayed by the higher temperature of 25° C., reaching only 12 per cent after 47 days at this temperature. No seedlings were produced at 30° C. Seeds held at 25° and 30° C. for 47 days were transferred to 20° C. which stimulated germination.

A sufficient number of seeds of polyploid *P. ovata* plants was not available for experimentation.

TABLE I
GERMINATION OF FRESHLY-HARVESTED SEEDS OF *PLANTAGO* SPP.
ON MOIST FILTER PAPER

Temperature (° C.)	Per cent germination after days											
	<i>P. rhodosperma</i>						<i>P. Wrightiana</i>					
	Diploid			Polyploid			Diploid			Polyploid		
	10	15	50	10	15	50	5	10	50	5	10	50
10	0	58	63	8	71	82	0	44	48	0	7	9
15	30	36	36	78	79	80	28	38	38	0	1	5
20	24	29	99	60	63	74	1	17	73	91	100	—
30	0	0	0	0	0	0	0	0	0	10	24	24
G.H.*	100	—	—	100	—	—	32	83	83	0	8	8
Lab.	0	0	1	1	1	11	0	0	31	15	41	65
5 to G.H.**	14	100	—	0	100	—	0	40	87	0	1	6
10 to G.H.**	100	—	—	79	85	87	0	100	—	0	60	60
10 to 20**	38	65	70	61	94	96	0	63	64	0	11	11
20 to 30**	5	8	83	25	25	78	12	15	29	86	92	100

* Greenhouse.

** Daily alternation.

Plantago rhodosperma

Plantago rhodosperma seeds are highly viable after four years of storage at room temperature. Nine days after planting seeds on moist filter paper 96 per cent germination was obtained at 20° C. Three other seedlings appeared at later dates giving a total of 99 per cent germination at 20° C. At an alternating temperature of 20° to 30° C., 91 per cent of the seeds had germinated in nine days and at the end of 22 days 100 per cent germination was obtained.

One-year-old seeds from polyploid plants (induced by soaking seeds in

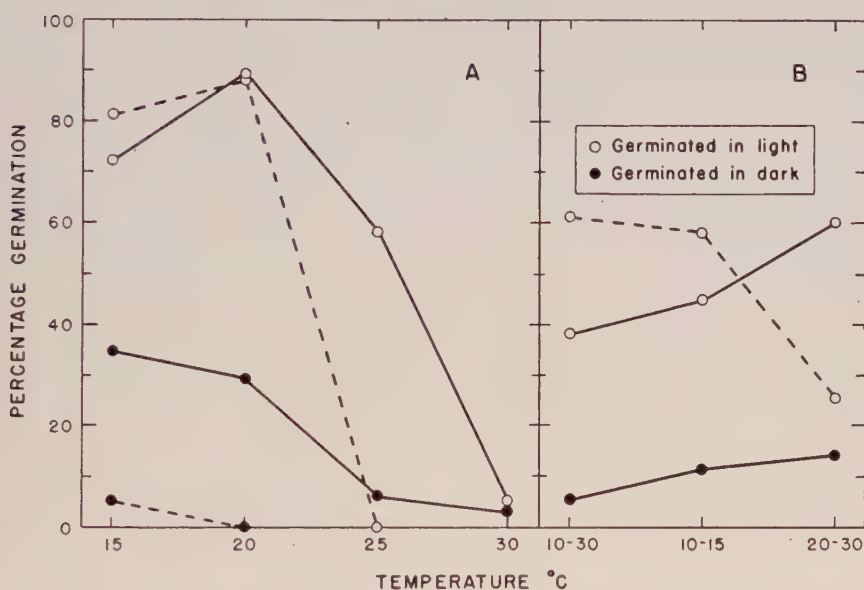


FIGURE 2. Germination of old and freshly-harvested seed from diploid plants of *Plantago rhodosperma*. Dotted lines indicate freshly-harvested seed and solid lines indicate old seed.

aqueous solutions of colchicine) germinated 100 per cent in the greenhouse and at a daily alternation of 5° C. to greenhouse.

Recently-harvested seeds of both diploid and tetraploid plants (Table I) were more specific in their germination requirements than old seeds. Both seed types responded well to all temperatures tried except those of 30° C. and the laboratory. The prompt and complete germination of seeds on moist filter paper in the greenhouse suggested the possible favorable effect of light. Further tests showed that seeds of this species are definitely light-sensitive as shown for diploid seeds in Figure 2. Old and fresh seed of the diploid gave 89 and 88 per cent germination in light at 20° C. while

in the dark the best germination was 35 and 5 per cent at 15° C. Seeds of the polyploid plants gave approximately the same reactions to light and the various temperatures.

The primary requirement for seed germination of *P. rhodosperma* appears to be light. However, some advantage is gained by a period of after-ripening in dry storage prior to germination. For example, one-year-old polyploid seeds germinated 100 per cent at 15° C. in light while fresh seeds germinated 49 per cent.

Plantago Wrightiana

Seeds stored for four years germinated 97 per cent at 20° C. and at a daily alternating temperature of 20° to 30° C. and germinated to a lesser degree over a wide range of temperatures. A comparison of germination of fresh diploid and polyploid seeds of this species is shown in Table I. It will be noted that polyploid seeds respond to higher temperatures than diploid seeds, as shown by the 24 and 5 per cent germination of the former at 30° and 15° C., respectively. However, 20° C. proved good for both seed types. There was an indication that diploid seeds favor light more than polyploid seeds as shown by the germination obtained on moist filter paper in the greenhouse. This was borne out by special light tests which indicated further that, though germination of seeds of *P. Wrightiana* was somewhat better in light than in dark, the reaction to light is not as pronounced as in seeds of *P. rhodosperma*.

Plantago amplexicaulis

All of the above methods for germination of *Plantago* seeds have been used for seeds of *P. amplexicaulis* without success. Seeds were viable, however, and after soaking for 24 hours in water the embryos could be dissected from the seed and grown on filter paper for a few days after which they could be planted in soil. Germination of these seeds is inhibited by the structures enveloping the embryos. However, preliminary tests with concentrated sulfuric acid failed to bring about germination of intact seeds.

SUMMARY

The germination requirements of three species of *Plantago* currently being used in genetic studies were investigated.

Dormancy of freshly-harvested seeds of *P. ovata* was expressed in their inability to germinate at a temperature as high as 30° C. and in their delay in germination at 20° C. as compared with old seeds. This dormancy was overcome by dry storage. Seeds of this species were not light-sensitive.

Germination of diploid and polyploid seeds of *P. rhodosperma* was favored by light and inhibited by high temperature (30° C.). Some

dormancy of freshly-harvested seed was shown but was not as marked as for *P. ovata*.

Polyploid seeds of *P. Wrightiana* tolerated higher germination temperatures than those of the diploid form while the latter appeared to be more light-sensitive than the former. A constant temperature of 20° C. proved good for the germination of both seed types.

Intact seeds of *P. amplexicaulis* failed to germinate under any of the conditions found favorable for the other three species, but excised embryos germinated readily.

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STUDIES ON THE NATURE OF FUNGICIDAL ACTION.
II. CHEMICAL CONSTITUTION OF BENZENOID AND
QUINONOID COMPOUNDS IN RELATION TO FUNGI-
TOXICITY AND INHIBITION OF AMINO- AND
SULFHYDRYL-DEPENDENT ENZYMES

ROBERT G. OWENS

INTRODUCTION

The fungitoxicity, bacteriostasis, and phytotoxicity of benzoquinones and naphthoquinones may be enhanced or impaired by substituents on the rings (7). It is not clear, however, whether the influence of substituents on biological activity results from changes in chemical reactivity or in physical properties associated with permeation and stability, or both. This paper reports initial results from a series of experiments designed to clarify this point.

A group of 27 compounds with benzenoid or quinonoid ring structures and with substituents of known inductive, resonance, and steric effects were tested for inhibition of fungus spore germination and for inhibition of pancreatic and malt amylases. Since amylases are highly sensitive to certain quinones (9), their inhibition provides a sensitive indication of changes in reactivity of quinones accompanying changes in their molecular constitution. Moreover, inhibition of these enzymes probably represents a rather general type of reaction applicable in a generic way to most enzymes dependent upon free sulfhydryl or amino groups for activity. Thus, the effects of molecular constitution of quinones on these enzymes may be comparable to effects on many enzymes dependent upon these groups and performing vital functions in metabolism.

Results show that inhibition of amylases is entirely consistent with the known chemical effects of substituents and with ring structure. However, while some general correlations between fungitoxicity and the tendency of the quinones to inhibit amylases can be made, specific compounds and even related classes of compounds are much more or much less fungitoxic than could be predicted on the basis of amylase inhibition. The results are discussed with reference to some general implications about the relative effects of chemical constitution of quinones on their chemical and physical properties and some possible contributions of these properties to *in vivo* toxicity. A preliminary report on this work has appeared elsewhere in abstract form (8).

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MATERIALS AND METHODS

Preparation of enzyme and test chemical systems and methods of assay of inhibition were described previously (9).

Spore germination inhibition was determined by the technique recommended by the committee on methods of the American Phytopathological Society (1, 2). Spores for these tests were taken from seven-day-old cultures of *Monilinia fructicola* (Wint.) Honey and *Alternaria oleracea* Milbraith grown on potato dextrose agar. All test chemicals were obtained from fungicide manufacturers¹ in purified form or from Distillation Products Industries Division of Eastman Kodak Company. All reagents were U.S.P. or C.P. grades.

RESULTS


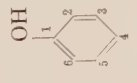


The effects of the chemicals on hydrolysis of starch by amylases were determined at a chemical concentration of 10^{-4} M. Previous tests (9) showed that for a specific quinone or hydroquinone at least a 100-fold increase in concentration is required to increase amylase inhibition from threshold to 100 per cent. Therefore, inhibition percentages for different compounds at 10^{-4} M indicated the relative activity of compounds whose potency differed roughly 10,000-fold with reference to concentration, since each increment of 1 per cent difference in inhibition represented an increment averaging about 1 per cent change in chemical concentration either higher or lower than 10^{-4} M. The wide range of chemical activities thus detectable at a single chemical concentration was considered satisfactory for the present purpose of activity comparisons of these compounds and eliminated the necessity for inhibition curve determinations.

The compounds were considered as derivatives of four different parent compounds indicated by formula in the first column of Table I. The substituents and numbers listed in column 3 refer to substituents and position of replacement of hydrogen on the parent nucleus. The ED₅₀ values shown for each of the compounds against *M. fructicola* and *A. oleracea* were derived by plotting spore germination percentages against concentration of the chemical on log probability paper.

Benzene derivatives (Group I, Table I) exhibited no activity that could be assigned to the benzene nucleus *per se*. Methyl, methoxy, or ethoxy benzenes were inactive against spores and were noninhibitory for amylases. Changes in the positions of methoxyl groups from the 1,2-positions, as in veratrole, to the 1,4-positions, as in hydroquinone-dimethyl ether, brought about no detectable change in activity against amylases or spores. Re-

¹ The author is indebted to the Naugatuck Chemicals Division of U. S. Rubber Company for purified samples of 2,3-dichloro-1,4-naphthoquinone and tetrachloro-*p*-benzoquinone.

TABLE I
EFFECTS OF CHEMICAL CONSTITUTION ON FUNGOTOXICITY AND INHIBITION OF PANCREATIC AND MALT AMYLASES

Group	Parent nucleus	Chemical	Substituents and carbon atom substituted	Enzyme inhibition per cent at $10^{-4}M$ of test chemical		Approximate ED_{50} ($\times 10^{-4}M$)
				Pancreatic amylase	Malt amylase	
I		Durene*	1,2,4,5- CH_3	0	0	>75
		4-Nitroveratrole	1,2- OCH_3 ; 4- NO_2	0	69	>54
II		Veratrole	1,2- OCH_3	3 (+)	0	>75
		Hydroquinone-dimethyl ether	1,4- OCH_3	0	0	>75
		Hydroquinone-diethyl ether	1,4- OC_2H_5	0	0	>60
		Hydroquinone*	1,4- OC_2H_5	1 (+)	1	>60
		2,4-Dichlorotoluene*	1- CH_3 ; 2,4- Cl	0	0	45
		3,4-Dichlorotoluene*	1- CH_3 ; 3,4- Cl	0	0	0.24
		Guaiacol	2- OCH_3	2 (+)	0	>60
		Hydroquinone-monomethyl ether	4- OCH_3	2 (+)	0	24
		Resorcinol	3-OH	2 (+)	0	>91
		Catechol	2-OH	6	0	5.5
III		Hydroquinone	4-OH	42	9	>91
		Di- <i>tert</i> -amylhydroquinone	4-OH; 2,5- <i>tert</i> -amyl	0	0	>40
		Toluhydroquinone	4-OH; 2- CH_3	25	19	9
		2,5-Dichlorohydroquinone	4-OH; 2,5- Cl	85	93	0.09
		<i>p</i> -Benzoquinone	—	92	96	0.65
		Tolu- <i>p</i> -benzoquinone	2- CH_3	79	69	1.00
		<i>p</i> -Xyloquinone	2,5- CH_3	14	14	0.10
		Chloranilic acid	3,6-OH; 2,5- Cl	1 (+)	0	11.00
		2,5-Dichloroquinone	2,5- Cl	97	99	0.03
		2,6-Dichloroquinone	2,6- Cl	95	99	0.10
IV		2,6-Dichloroquinonechloroimide	4- NC ; 2,6- Cl	86	99	0.05
		Tetrachloro- <i>p</i> -benzoquinone*	2,3,5,6- Cl	97	100	0.04
		1,4-Naphthoquinone	1,2- O ; 4-H	28	72	0.08
		1,2-Naphthoquinone	2- CH_3	44	96	0.89
		2-Methyl-1,4-naphthoquinone*	2,3- Cl	1 (+)	0	0.08
		2,3-Dichloro-1,4-naphthoquinone*	—	57	70	0.001
		—	—	—	—	0.002
		—	—	—	—	0.06
		—	—	—	—	1.50
		—	—	—	—	0.09

* Compounds not completely soluble at $10^{-4}M$.
(+) Indicates increase in activity over that of controls.

placement of hydrogen in the 4-position of veratrole by a nitro group, however, resulted in selective activity. 4-Nitroveratrole was moderately inhibitory for malt amylase but noninhibitory for pancreatic amylase. Similarly, it was moderately inhibitory for spores of *M. fructicola* but inactive even at the highest concentration tested against *A. oleracea*.

Since none of the other compounds exhibited such wide differences in activity against the two amylases the possibility that pH might control the tendency of 4-nitroveratrole to react was examined. This was suggested by the fact that the systems in which malt and pancreatic amylase activity was determined were regulated at pH 4.6 and 7.0, respectively. Therefore, the pH of suspensions of spores, mycelium and a few millimeters of agar from beneath the mycelial mat of two-week-old agar cultures of *M. fructicola* and *A. oleracea* was determined. The pH values obtained were 4.8 to 4.9 for *M. fructicola* and 6.2 to 6.3 for *A. oleracea*. These results indicated that pH might be a controlling factor in the specificity of fungicidal action of certain compounds and further experiments on this point are being carried out.

Both isomers of dichlorotoluene used were active against spores of the two fungi. Positional effects of chlorine atoms on fungitoxicity were marked. 2,4-Dichlorotoluene exhibited a low order of fungicidal activity whereas 3,4-dichlorotoluene was highly active. Both derivatives were inactive against amylases. However, true solutions containing 10^{-4} M of these compounds could not be obtained because of their extremely low solubility in water, so that positional effects on chemical reactivity could not be determined by means of amylase reactions.

In tests already reported (9) phenol at 10^{-4} M did not inhibit either pancreatic amylase or malt amylase. Further tests on the effects of substituents on the inhibitory action of phenolic compounds (Group II) showed profound electromeric effects on the inhibitory properties of these compounds. Some anomalies between their reactions with the amino or sulfhydryl groups of the amylases and spore germination inhibition were indicated also. Methoxy-phenols did not inhibit either amylase. Change in the position of the methoxyl group from the *ortho* position, in guaiacol, to the *para* position, in hydroquinone-monomethyl ether, produced no measurable change in activity against amylases but resulted in some inhibition of spores of both fungi.

Positional effects of hydroxyl groups were exemplified by resorcinol, catechol, and hydroquinone. Resorcinol, with 1,3-arrangement of hydroxyl groups, was inactive against fungus spores and amylases. Catechol and hydroquinone were slightly or moderately inhibitory for pancreatic amylase but less active against malt amylase. Of the two compounds, hydroquinone, with 1,4-hydroxyl groups, was somewhat more inhibitory for amylases than was catechol, with 1,2-hydroxyl groups. However, the

order of effectiveness was reversed in case of spore germination inhibition. Hydroquinone was selectively active against *M. fructicola* whereas catechol was somewhat active against spores of both species of fungi. Antifungal action and enzyme inhibition seemed to be correlated with hydroxylation in positions which permitted quinone or semiquinone formation.

Known electromeric effects of substituents were reflected consistently by the inhibition of pancreatic amylase. The methyl group of toluhydroquinone, which contributes to the electron density of the ring and the oxygen-hydrogen bond of the hydroxyl group, decreased activity of the compound against pancreatic amylase. The chlorine atoms of 2,5-dichlorohydroquinone, which decrease the electron density of the ring and the oxygen-hydrogen bond of the hydroxyl groups, increased activity of the compound against pancreatic amylase. The reduced activity of both toluhydroquinone and catechol was consistent with the expected steric hindrance and intramolecular hydrogen bonding arising from *ortho* methyl and hydroxyl groups.

The reactions with malt amylase were not so amenable to interpretation on the basis of electromeric or steric effects. Toluhydroquinone was slightly more active than hydroquinone, so that the influence of electron density of the ring was counteracted by other influences, possibly pH of the systems. The fact that neither hydroquinone nor catechol was as active against malt amylase as against pancreatic amylase suggested that the reactivity was associated with the ability of the hydroxyl groups to dissociate, which would be reduced in the acid medium of malt amylase systems.

Replacement of hydrogen by two *tertiary* amyl groups in positions adjacent to the hydroxyl groups of hydroquinone destroyed activity as an enzyme inhibitor or a spore germination inhibitor. This suggests that dissociation of the hydroxyl groups and the approaches to the unsubstituted *ortho* positions were blocked by steric hindrance.

Quinones (Group III) were more active as inhibitors of amylases and of spore germination than were the corresponding hydroquinones. Substituent effects, however, were similar. Unsubstituted benzoquinone was highly inhibitory for spores of both fungi and for both amylases. Activity against amylases and fungus spores was reduced upon replacement of hydrogen by one methyl group. Electronic and steric effects thus corresponded to effects of the methyl group of toluhydroquinone. Activity against amylase was further reduced upon substitution by a second methyl group to form xyloquinone. However, inhibition of fungus spores was increased, so that electronic and steric effects were probably not the primary controlling factors in fungitoxicity of this compound. Activity against amylases was entirely destroyed by hydroxyl groups adjacent to the carbonyl groups even though chlorine atoms were present on the ring,

q.v. chloranilic acid. Some fungitoxicity persisted but was markedly reduced. Without *ortho* hydroxyl groups chlorination increased enzyme inhibition and spore germination over the unsubstituted benzoquinone. 2,6-Dichloroquinone appeared to be somewhat less effective as a spore germination inhibitor than other chlorinated derivatives. Other experiments suggested that this may be due to a more rapid rate of breakdown of 2,6-dichloroquinone in solution. Tetrachloroquinone was most effective of the chlorinated derivatives against *A. oleracea* whereas 2,5-dichloroquinone appeared to be most effective against *M. fructicola*. Generally, the increase in number of chlorine atoms to four did not result in proportionally as great an increase in fungitoxicity as the first two chlorine atoms substituted for hydrogen on the benzoquinone nucleus. Positional effects were apparently associated with stability.

In general, a particular substituent on the benzoquinone and hydroquinone nuclei influenced inhibition of amylases by these compounds in a corresponding manner. Chlorination invariably increased amylase inhibition while hydroxylation and methylation decreased inhibition. Thus the same electronic and steric effects appeared to be operative whether the co-functional group was hydroxyl or carbonyl. In either case the quinonoid structure was essential for high activity as shown by the lack of enzyme inhibition by compounds incapable of forming quinones and with the oxygen atom involved in an undissociable ether linkage. The functional character of the quinonoid structure was further indicated by the fact that benzoquinones were much more active than the corresponding hydroquinones.

The fungicidal action of naphthoquinones (Group IV) was highly anomalous with respect to amylase inhibition. They were much less inhibitory for either amylase than similarly substituted benzoquinones, yet with the exception of 1,2-naphthoquinone, all derivatives were much more fungitoxic. On the other hand, the effects of substituents were closely analogous to substituent effects on benzoquinones. Unsubstituted 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone were about equally toxic to spores, whereas the latter was much less inhibitory for amylases, possibly due to its relatively slight solubility. Replacement of hydrogen by chlorine in the 2- and 3-positions increased fungitoxicity over that of other derivatives. True relative values for amylase inhibition could not be obtained at $10^{-4}M$ because of unequal solubility of derivatives, but in previous tests (9) it was shown that chlorination increased enzyme inhibition also. 1,2-Naphthoquinone was less than one-tenth as active as 1,4-naphthoquinone against fungus spores but was slightly more active against amylases.

A number of compounds listed in Table I exhibited activity against fungus spores entirely anomalous to the activity expected on the basis

TABLE II
MOLECULAR CONFIGURATIONS OF COMPOUNDS SHOWING MARKEDLY DIFFERENT
ACTIVITY AGAINST FUNGUS SPORES AND AMYLASES

Relatively greater activity against		
Fungus spores		Amylases

of amylase inhibition. The molecular constitutions of these compounds are shown in Table II. *p*-Xyloquinone was as fungitoxic as 2,6-dichloroquinone despite the steric effects of methyl group or the fact that inductive effects of methyl and chlorine groups are opposite. All 1,4-naphthoquinone derivatives were highly fungitoxic but at best only moderately inhibitory for amylases. The toluene derivatives showed no activity against amylases but were slightly to highly active against spores. On the other hand, 1,2-naphthoquinone was much less fungitoxic than 1,4-naphthoquinone derivative but more active than 1,4-naphthoquinone against amylases. Tetrachlorobenzoquinone was found previously (9) to be extremely active against amylases but was relatively little more effective against spores than 2,5-dichloroquinone. Thus, the principal anomalies between reactions of the types occurring between many of the quinones and amylases appear to be related to properties associated with methylation and with the bicyclic structure of 1,4-naphthoquinones.

DISCUSSION

There are many points of similarity between the inhibition of amylases by quinones reported in this paper and inhibition of carboxylase reported

by Kuhn and Beinert (6) and Foote, Little, and Sproston (4). Halogenation increased activity consistently whereas alkylation or hydroxylation generally reduced activity. These relationships were so closely correlated with toxicity to spores that Foote, Little, and Sproston (4) suggested that carboxylase inhibition might be used as a preliminary screen for fungicides. However, there are enough discrepancies between the action against enzymes *in vitro* and living spores, revealed by the present study and by the review by McNew and Burchfield (7), to indicate that the enzyme test might be misleading.

Sulphydryl- and amino-dependent enzymes are the most sensitive types to quinones yet studied. This suggests that discrepancies between reactivity, as indicated by inhibition of these enzymes, and fungitoxicity may be due to physical properties related to the physical and chemical make-up of the fungus spores. The data show that halogenation *per se* or reactivity alone does not account satisfactorily for fungitoxic action since the nonsubstituted 1,4-naphthoquinone is almost as active against spores as chlorinated benzoquinones, but much less active against amylases. This indicates that properties arising from ring structure contribute to fungitoxicity even though the rate of reaction with amylases is adversely affected. Burchfield and McNew (3) have pointed out the photolability of some quinones. Photochemical decomposition decreases with decreasing oxidation potential. Since the 1,4-naphthoquinones have a large ring system which increases resonance energy, their decomposition rates are considerably less than those of benzoquinones. Thus, it appears that fungitoxic properties are improved by an appropriate balance between the rates of decomposition and reactivity and that the balance may sometimes be achieved by changes in ring constitution.

Fungitoxicity is not dependent only on the quinonoid ring structure, however, since both isomers of dichlorotoluene were active against fungus spores. Neither was active against amylases, which may have been due to the slight solubility of the compounds in water. Thus, in order to account for their fungitoxicity it is necessary to visualize some hypothetical mechanism of water solubilization of the compounds, in which case they would be changed chemically, or to assume that they exert their influence in or at the interfaces of lipid structures. The latter seems more likely and involves the concept of Horsfall and Rich (5) who attribute activity against fungi to two types of groups, e.g., a reactive group and a lipid soluble group. While this concept is of necessity very general to include many classes of fungicidal compounds, the present data seem to agree with it in principle. Lipid solubility of many classes of compounds, however, must be attributed to the molecule as a whole rather than to a chemical group.

Discrepancies between fungitoxicity and amylase inhibition of several

other compounds require some explanation. The selective action of 4-nitroveratrole has already been attributed to pH differences of the enzyme systems and spore environment of the two species. *p*-Xyloquinone exhibited activity against spores equal to that of 2,6-dichloroquinone. Since the substituents are of opposite electromeric effects and reactivity with amylases are markedly different, other properties must be involved. Other methylated quinones (toluhydroquinone, tolu-*p*-quinone, and 2-methyl-1,4-naphthoquinone) were relatively more active against fungus spores than against amylases. Since methylation lowers the oxidation potential of quinones, it would be expected to decrease the rate of photochemical decomposition. Methyl groups also increase lipid solubility, so that the results obtained are entirely consistent with the known influence of these groups.

The question arises as to whether the fungitoxicity is predominantly limited by physical or chemical properties of the molecule. A consideration of all compounds studied with regard to ring structure and substituent groups leaves little doubt that both physical and chemical properties contribute to fungitoxicity and that either may predominate, depending upon the nature of the ring and substituents.

SUMMARY

1. The variations in fungitoxicity and inhibition of pancreatic and malt amylases accompanying changes in molecular constitution of 27 benzenoid and quinonoid compounds were determined. The compounds had been selected to represent benzenoid, benzoquinonoid, and naphthoquinonoid ring structures and to indicate the effects of various substituents with known resonance, inductive, and steric effects on the relative biological activities of these compounds.

2. Benzenoid compounds, structurally incapable of oxidation to quinones or semiquinones in solution, were inactive against amylases but specific compounds were active against spores of *Monilinia fruticola* and *Alternaria oleracea*.

3. Hydroquinones, benzoquinones, and naphthoquinones usually inhibited both enzymes. Enzyme inhibition was entirely consistent with known steric, inductive, and resonance effects of substituents. Substituents always influenced the inhibitory activity of the hydroquinones, benzoquinones, and 1,4-naphthoquinones in a corresponding manner. Substitution of hydrogen by chlorine increased inhibition, whereas methylation or hydroxylation decreased inhibition.

4. Fungitoxicity was increased by chlorination of benzoquinone, 1,4-naphthoquinone and hydroquinone in a manner corresponding to the increase in amylase inhibition. However, methylation usually enhanced or failed to affect fungitoxicity although it reduced amylase inhibition.

5. 1,4-Naphthoquinones were much more fungitoxic than the corresponding benzoquinones whereas the latter were much more inhibitory for amylases.

6. The possible influence of the larger ring structure of 1,4-naphthoquinones and of methylation of benzoquinones on chemical stability and permeation of spores is discussed as a possible basis for discrepancies between the relative fungitoxic action of these compounds and their inhibition of amylases.

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ACCUMULATION OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM BY FUNGUS SPORES IN MIXED AND CONSECUTIVE TREATMENTS¹

LAWRENCE P. MILLER, S. E. A. MCCALLAN, AND RICHARD M. WEED

Studies with radioactive tracers have shown that 2-heptadecyl-2-imidazoline, silver, and cerium are taken up rapidly and in large amounts when fungus spores of various species are suspended in relatively low concentrations of these toxicants (5). The uptake of large quantities on a spore weight basis was necessary before germination was adversely affected. ED₅₀ values for the imidazoline, for example, were found to be as high as 5000 to 9000 p.p.m. If these quantities are primarily deposited on cell surfaces, the layers would have to be several molecules thick to account for the amounts involved. It seemed of interest, therefore to determine, for example, whether previous treatment with 2-heptadecyl-2-imidazoline adversely affected the uptake of a second toxicant and whether a second toxicant caused some of the imidazoline previously taken up to be released. It was already known that in general the toxicants being studied were held quite firmly by the spores and were not released on resuspension in distilled water but only after rather drastic treatment. Also the affinity of the spores of various species for the toxicants was known to be determined largely by factors other than the amount of cell surface per unit weight as determined by the relative sizes of the spores involved.

To study the possible role of sites of accumulation in the uptake of these fungicides, extensive tests were carried out in which spores of various species of fungi were treated either consecutively with the imidazoline, silver, and cerium or simultaneously with mixtures of two or all three. Techniques were employed by which it was possible to determine C¹⁴, Ag¹¹⁰, and Ce¹⁴⁴ all in the same sample and therefore interactions as to uptake and release among these materials could be followed quantitatively.

The results indicate that the three toxicants being studied act independently and that the uptake of one is not affected by the others. This holds true even if the spores are already saturated by one or the other of the fungicides.

MATERIALS AND METHODS

FUNGUS SPORES

Conidia from the following species of fungi were used: *Monilinia fructicola* (Wint.) Honey (formerly called *Sclerotinia fructicola*), *Glomerella*

¹ These investigations were conducted in cooperation with the United States Atomic Energy Commission, Contract AT(30-1)-788.

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cingulata (St.) Sp. & von S., *Aspergillus niger* van Tiegh, *Neurospora sitophila* (Mont.) Shear & Dodge, and *Venturia pyrina* Aderh. Methods employed for culturing the fungi, harvesting the spores, determining the spore weights, and the nutrients added for germination tests are given in earlier papers (4, 5).

LABELED FUNGICIDES

The 2-heptadecyl-2-imidazoline used had the labeled carbon in the 2-position in the ring (5). Stock solutions were maintained in acetone and for the tests usually 0.2 ml. was added to 10 to 12 ml. of a spore suspension. The silver was applied as silver nitrate with enough Ag^{110} , obtained from a preparation from the Oak Ridge National Laboratory with a specific activity when received of 28.1 mc. per gram, added to give between 500 to 750 counts per minute per microgram of silver. Cerium was used as $\text{Ce}_2(\text{SO}_4)_3 \cdot 8\text{H}_2\text{O}$ with Ce^{144} supplied from carrier free $\text{Ce}^{144}\text{Cl}_3$ obtained from the Oak Ridge National Laboratory. The amount of Ce^{144} added to the preparations was adjusted to give about 500 to 750 counts per minute per 10 micrograms of cerium. In experiments in which movement of SO_4^{--} was also being followed S^{35} labeled sulfate was added.

The concentrations usually employed were about 3 p.p.m. with the imidazoline, 1 p.p.m. with silver, and 10 p.p.m. with cerium. These concentrations were chosen because of differences in the fungitoxicity of the materials and also because much information was available from previous experiments on the rate of uptake and toxicity of these fungicides when used at these concentrations. Some experiments were carried out in which concentrations differing quite widely from those indicated were employed. Effective doses were further modified by varying the quantities of spores present in the tests.

EXPOSURE OF SPORES TO TOXICANTS

In the tests a known quantity of spores was suspended in a 15-ml. centrifuge tube with a conical bottom in a volume of 10 to 12 ml. of distilled water containing the desired quantity of fungicide or fungicides. After the spores had been in suspension for a given length of time the mixture was centrifuged. Upon centrifugation the spores were rapidly deposited at the bottom of the tube and were not considered as being exposed to the fungicides until again resuspended throughout the solutions. Amount of toxicant taken out of solution was determined by taking samples, usually 1 ml., after various time intervals. The sample taken was evaporated to dryness in a small planchet under a heat lamp and the radioactivity determined as indicated below. The amount taken up could then be determined from the loss in activity. The amount of toxicant taken up by the spores could also be determined directly by determining the radioactivity of a

suitable aliquot and correcting for any radioactivity in the supernatant in which the spores were suspended. When spores were included in the sample to be counted, the quantity was kept low enough so that there was no significant effect from self-absorption.

If the spores were to be exposed to a second fungicide, this was either added to the supernatant or the supernatant was removed with the aid of a pipette and fresh solution added. In studying the elution or extraction of fungicide which had been taken up by the spores the extractant to be used was added to the bottom of the tubes, thoroughly mixed with the spores and removed with a pipette after centrifugation. In these procedures care had to be taken not to withdraw any spores with the pipette as supernatant was being removed. A few drops of liquid above the spores, as well as some held between the tightly packed spores, would remain after each withdrawal and become part of subsequent samples. However, the effect of this was not serious enough to interfere in the interpretation of the results obtained.

QUANTITATIVE DETERMINATION OF INDIVIDUAL RADIOISOTOPES IN MIXTURES OF C^{14} , Ag^{110} , Ce^{144} , AND S^{35}

Radioactivity was determined by the use of conventional scaling equipment and end window type Geiger tubes with thin windows. The radioactive fungicides were available with sufficient specific activity and effective against fungi at such low concentrations that measurements could be made on the samples and on spores without significant interference from self-absorption. Samples to be counted were placed in stainless steel, nickel-plated, or glass planchets one inch in diameter. An automatic sample changer was used and was usually set to measure the time required to give 1024 counts. Usually at least three and frequently more such measurements were made on each sample.

When it was necessary to determine individual isotopes in mixtures, the counting was carried out with aluminum absorbers in the system in addition to the counting without absorbers. As a routine, two absorbers were used, one with a thickness of 13.0 mg. per sq. cm. and the other with a thickness of 27.5 mg. per sq. cm. With the particular Geiger tube used in these tests and the geometry of the counting system the first absorber gave counts for Ce^{144} , Ag^{110} , C^{14} , and S^{35} equal to 74, 58, 2.5, and 3.1 per cent, respectively, of that obtained without an absorber. With the thicker absorber 65 and 39 per cent of the counts obtained in the absence of an absorber for Ce^{144} and Ag^{110} , respectively were obtained and C^{14} and S^{35} gave no counts. When mixtures of three isotopes were present in a sample it was necessary to have values for the activity with both absorbers and without any absorber and to solve the three simultaneous equations, which could then be set up. When only two isotopes were to be separated, the values

with only one absorber are necessary but usually in these instances also two absorbers were used. It was then possible to obtain two values for a given isotope and average the results for a somewhat more accurate estimate.

It was not possible to determine separately S^{35} and C^{14} in a mixture with the aid of absorbers. But advantage could be taken of the large differences in half life between these two isotopes and the determinations without absorber repeated after a significant loss in activity of S^{35} . Then simultaneous equations based on the decay during the time interval between the two determinations could be used to give the quantities of the two isotopes present.

RESULTS

CONSECUTIVE TREATMENTS WITH VARIOUS TOXICANTS

The data in Table I illustrate the effect of previous treatment with 2-heptadecyl-2-imidazoline on the subsequent uptake of cerium by spores of four species of fungi. In this experiment 10 mg. each of spores of *Neurospora sitophila*, *Monilinia fruticola*, *Glomerella cingulata*, and *Aspergillus niger* were exposed to 10 ml. of aqueous solutions containing 3.22 p.p.m. of 2-heptadecyl-2-imidazoline. The uptake was determined after two minutes and then another 10 mg. of spores was added and uptake determined after

TABLE I
QUANTITIES OF CERIUM TAKEN UP BY SPORES OF FOUR SPECIES OF FUNGI AS INFLUENCED BY THE PREVIOUS UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE

Species	Spore wt., mg.	Imidazoline previously taken up, micrograms	Uptake of cerium* in micrograms after 2 and 40 minutes	
			2	40
<i>Neurospora sitophila</i>	50	58.2	18.1	24.0
<i>Monilinia fruticola</i>		49.2	38.6	39.4
<i>Glomerella cingulata</i>		49.3	9.7	11.9
<i>Aspergillus niger</i>		20.7	89.3	94.7
<i>Neurospora sitophila</i>	10	0	7.5	9.5
<i>Monilinia fruticola</i>			14.8	18.0
<i>Glomerella cingulata</i>			5.1	5.7
<i>Aspergillus niger</i>			48.0	47.4

* Quantity available, 100 micrograms.

another two minutes. This was continued until a total of 50 mg. of spores had been added for each species. Then a further 32.2 micrograms of the imidazoline was added and the uptake determined after another four minutes of exposure. If the spores had taken up all the imidazoline presented before any was removed by sampling, they would have taken up a total of 64.4 micrograms. The data in column three of Table I show that from 20.7

to 58.2 micrograms was removed from the solution, depending upon the species involved. (Details of the uptake during these treatments with the imidazoline are given in Table VI of a previous paper [5].) On subsequent exposure to 100 micrograms of cerium, uptake as shown in the last column of Table I was obtained. By far the largest amount was taken up by spores of *Aspergillus niger*, the least by spores of *Glomerella cingulata*, while spores of *Monilinia fructicola* and *Neurospora sitophila* took up quantities intermediate between these two extremes. In another experiment 10-mg. lots of spores of these four species were exposed to 100 micrograms of cerium without previous treatment with the imidazoline. The data, given in the lower

TABLE II
EFFECT OF PREVIOUS UPTAKE OF SILVER ON THE AMOUNT OF 2-HEPTADECYL-2-
IMIDAZOLINE TAKEN UP IN 10 MINUTES BY SPORES OF
VENTURIA PYRINA

Spore wt., mg.	Ag taken up, p.p.m.	Imidazoline and silver content in p.p.m. after subsequent treatment with the imidazoline	
		Imidazoline	Silver
1.4	1394	7300	1014
2.5	None	7000	—
3.4	1324	4188	1059
5.0	None	5180	—
7.4	1163	2527	1020
10.0	None	2200	—
15.5	791	1116	729
20.0	None	1260	—
31.5	389	638	350
40.0	None	588	—
63.4	221	404	185
80.0	None	291	—

part of Table I, show that the relative affinity of the spores for cerium was the same whether or not previous exposure to the imidazoline had taken place.

Experiments in which spores of *Venturia pyrina* were treated first with silver and then with 2-heptadecyl-2-imidazoline are summarized in Table II. The silver which had been taken up varied from about 1400 p.p.m. of spore weight to only 200 p.p.m. with the larger lot of spores. When such spores were treated with 2-heptadecyl-2-imidazoline and the amount taken up was compared with that taken up by similar quantities of previously untreated spores, the data of column three of the table were obtained. The quantity of the imidazoline taken up was essentially the same whether or not the spores had previously taken up silver. The uptake of the imidazoline did not result in an appreciable release of the silver previously taken up.

An experiment was carried out with spores of *Neurospora sitophila* in which four lots were exposed to imidazoline, silver, cerium, and silver, re-

spectively, and the uptake determined after a five-minute period. 2-Heptadecyl-2-imidazoline was then added and the uptake determined as well as that of any toxicants remaining from the first treatment. The remaining supernatant was then removed and further treatments, as indicated in

TABLE III

UPTAKE AND RETENTION OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM BY SPORES OF *NEUROSPORA SITOPHILA* WHEN EXPOSED IN CONSECUTIVE TREATMENTS IN VARIOUS ORDERS FOR 5-MINUTE PERIODS

Lot No.	Exposure			Quantities in spores after treatments, micrograms		
	No.	Toxicant	Quantity present, micrograms	Imidazoline	Silver	Cerium
A	1	Imidazoline	32.2	23.5		
	2	Imidazoline	32.2	47.9		
	3	Imidazoline	32.2	79.3		
	4	Imidazoline	32.2	107.8		
	5	Imidazoline	32.2	132.2		
	6	Imidazoline	32.2	153.8		
B	1	Silver	10.4		8.5	
	2	Imidazoline	32.2	19.2	8.0	
	3	Silver	10.4	19.2	15.4	
	4	Imidazoline	32.2	33.3	14.1	
	5	Silver	10.4	32.0	22.6	
	6	Imidazoline	32.2	53.3	22.6	
C	1	Cerium	100.0			34.3
	2	Imidazoline	32.2			32.2
	3	Cerium	100.0	27.9		36.8
	4	Imidazoline	32.2	55.7		36.8
	5	Cerium	100.0	54.2		39.8
	6	Imidazoline	32.2	78.3		47.8
D	1	Silver	10.4		8.5	
	2	Imidazoline	32.2	20.0	8.4	
	3	Cerium	100.0	20.0	8.4	42.2
	4	Imidazoline	32.2	48.0	8.4	48.0
	5	Silver	10.4	46.9	16.6	49.2
	6	Imidazoline	32.2	77.6	17.7	49.2

Table III, were carried out. The data do not indicate any appreciable effect of previous treatment on uptake of a different toxicant. There is also no evidence that the addition of a second or third toxicant brings about a release of toxicant previously taken up.

SIMULTANEOUS EXPOSURES TO VARIOUS TOXICANTS

Possible interactions in the rapid uptake of various toxicants by fungus spores were studied further by exposing spores of a number of species simultaneously to mixtures of 2-heptadecyl-2-imidazoline, silver, and cerium, and comparing the uptake with that obtained when the toxicants were used individually. The results are summarized in Tables IV, V, and VI.

TABLE IV

UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM,
AND MIXTURES OF THESE TOXICANTS BY SPORES OF
NEUROSPORA SITOPHILA (10-MG. LOTS)

Toxicant	Micrograms in 10 ml.	Cumulative uptake in micrograms after various time intervals in minutes			
		0.5	1.5	36.5	171.5
Imidazoline	32.2	2.7	9.8	14.7	16.8
Silver	10.4	6.1	7.4	8.3	8.1
Cerium	100.0	9.3	11.5	6.2	11.0
Imidazoline Silver	32.2 10.4	4.9 5.7	9.8 6.4	14.0 6.7	15.4 9.1
Imidazoline Cerium	32.2 100.0	6.8 3.5	13.9 1.7	22.2 5.6	25.1 13.2

The experiments were carried out with spores of *Neurospora sitophila*, *Aspergillus niger*, and *Glomerella cingulata*. Spores of these species were known to differ in their affinity for the toxicants being used, especially in regard to 2-heptadecyl-2-imidazoline and cerium. The data do not indicate any marked effect of the toxicants upon each other. For example, uptake of large amounts of cerium by spores of *Aspergillus niger* (Table V) did not affect the amount of the imidazoline or silver taken up at the same time. Nor did spores which were exposed to considerably more silver and imidazo-

TABLE V

UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM, AND
MIXTURES OF THESE TOXICANTS BY SPORES OF *ASPERGILLUS*
NIGER (10-MG. [DRY WT.] LOTS)

Toxicant	Micrograms in 12 ml.	Cumulative uptake in micrograms after various time intervals in minutes				
		1	6	21	75	195
Imidazoline	31.1	15.6	18.3	20.7	22.1	22.5
Silver	4.72	2.3	2.4	2.5	2.8	2.8
Cerium	45.4	42.6	43.9	44.1	44.3	43.6
Imidazoline Silver	31.1 4.72	9.9 2.5	18.4 2.7	17.3 2.6	25.0 2.1	21.8 2.9
Imidazoline Cerium	31.1 45.4	15.6 42.4	20.1 44.5	21.5 45.1	23.7 44.1	23.4 44.2
Imidazoline Silver Cerium	31.1 4.72 45.4	13.5 1.9 42.7	20.2 1.3 45.2	21.7 2.7 41.9	21.9 2.7 41.9	23.6 2.7 43.8

line than they took up during the experimental period, take up any less cerium than when cerium was used alone. A similar lack of interactions is evident in the results of tests with *Glomerella cingulata* and *Neurospora sitophila* as shown by the data in Tables IV and VI.

TABLE VI
UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM,
AND MIXTURES OF THESE TOXICANTS BY SPORES OF
GLOMERELLA CINGULATA (50-MG. LOTS)

Toxicant	Micrograms in 11 ml.	Cumulative uptake in micrograms after various time intervals in minutes		
		1	6	21
Imidazoline	29.2	15.1	17.9	20.3
Silver	4.5	4.2	4.3	4.2
Cerium	45.2	14.5	28.0	32.1
Imidazoline Silver	29.2 4.5	17.0 4.1	19.9 4.4	29.8 4.3
Imidazoline Cerium	29.2 45.2	21.5 19.5	27.1 36.1	25.3 38.7
Imidazoline Silver Cerium	29.2 4.5 45.2	21.6 4.2 15.0	24.7 4.5 28.7	* * *

* Total number of counts at this sampling period was so low that determinations of activity ascribed to C^{14} , Ag^{110} , and Ce^{144} were not feasible.

Behavior on Further Treatment

Results obtained when spores, which had already taken up large amounts of one, two, or three of the toxicants being considered, were exposed further to additional quantities, are shown in Tables VII and VIII. The spores used to obtain the data of Table IV were treated further as indicated in Table VII and those covered in Table V were handled as shown in Table VIII. With these spores considerable toxicant had already been taken up and effects of the uptake of one toxicant upon that of another would be expected to become more pronounced as saturation or near saturation is reached with one or more of the toxicants being studied. It will be noted that although spores of *Aspergillus niger* took up only a little over half of the silver presented to them as shown in the data of Table V, on further exposure to a higher concentration (about twice as high as that remaining at the end of the previous experiment) additional quantities were taken up. Uptake was slower, however, than when previously untreated spores were used. Even under these conditions uptake of the various toxicants was independent of the presence of previous uptake of other toxicants.

TABLE VII
RATE OF UPTAKE OF VARIOUS TOXICANTS BY SPORES OF *NEUROSPORA*
SITOPHILA (10-MG. LOTS) WHICH HAD BEEN TREATED PREVIOUSLY

Taken up before start of test		Additional toxicant in supernatant, micrograms	Amount taken up after various time intervals in minutes		
Toxicant	Micrograms		1	6	36
Imidazoline	16.8	21.9	0.6	6.4	7.9
Silver	8.1	6.2	3.3	4.5	4.7
Cerium	11.0	93.7	-1.3	3.6	4.2
Imidazoline	15.4	23.0	5.8	9.4	12.9
Silver	9.1	7.1	3.5	4.5	4.7
Imidazoline	25.1	16.9	-3.1	-8.6	8.5
Cerium	13.2	90.3	7.6	32.9	16.7

The results with spores of *Neurospora sitophila* also indicate a lack of interdependence of the uptake of one toxicant upon another. There is some indication that the uptake of cerium is slightly higher in the presence of 2-heptadecyl-2-imidazoline than in its absence as shown by data in Tables IV and VII. Other experiments not reported in the present paper support this conclusion and this point warrants further study.

EFFECT OF TERGITOL 7 ON UPTAKE OF TOXICANTS

Tests with the surface active agent, Tergitol 7 (25 per cent sodium heptadecyl sulfate in aqueous solution), have shown it to interfere considerably with the uptake of 2-heptadecyl-2-imidazoline by fungus spores.

TABLE VIII
RATE OF UPTAKE OF VARIOUS TOXICANTS BY SPORES OF *ASPERGILLUS*
NIGER ON FURTHER TREATMENT

Taken up before start of test		Additional toxicant, micrograms in 12 ml.	Amount taken up after various time intervals in minutes				
Toxicant	Micrograms		1	6	21	1071	5331
Silver	2.8	5.3	0.74	1.3	1.5	2.4	2.7
Cerium	43.6	50.2	37.2	40.3	39.4	38.4	40.9
Imidazoline	21.8	34.9	-0.1	7.9	7.4	14.2	14.4
Silver	2.9	5.3	0.7	1.3	1.7	2.3	2.6
Imidazoline	23.4	34.7	5.1	12.4	15.2	20.2	20.3
Cerium	44.2	50.1	31.6	38.3	40.2	40.3	43.5
Imidazoline	23.6	34.7	7.6	11.7	13.8	16.8	17.8
Silver	2.7	5.3	0.9	1.1	1.8	3.0	3.3
Cerium	43.8						

Tergitol 7 retarded the uptake both when the spores were exposed to it before treatment with the imidazoline and when the Tergitol 7 and the imidazoline were used simultaneously. In Table IX are reported results of a test in which 12.5 and 25.0 mg. of Tergitol 7 were added to 10 and 90 mg., respectively, of spores of *Neurospora sitophila* suspended in 10 ml. of water. The mixture was centrifuged promptly, the supernatant removed, the spores resuspended in 10 ml. of water, the mixture again centrifuged and the supernatant removed. After the addition of another 10 ml. of water, ex-

TABLE IX
EFFECT OF TERGITOL 7 ON UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE
(32.2 MICROGRAMS IN 10 ML.) BY SPORES
OF *NEUROSPORA SITOPHILA*

Spore wt., mg.	Tergitol 7 used*	Cumulative uptake in micrograms after various time intervals in minutes			
		1	6	156	876
10	No	7.3	17.4	21.9	22.6
10	Yes	1.3	7.6	9.6	10.4
30	No	19.8	23.0	26.2	26.7
90	No	21.9	24.3	26.7	27.1
90	Yes	6.1	6.3	9.3	12.4
Water control	No	7.5	—	14.2	19.3

* Tergitol 7 was added to 10 ml. of spore suspension and then the mixture was centrifuged and the supernatant removed. The spores were then resuspended in another 10 ml. of water, the mixture centrifuged, and the supernatant again removed. After a further addition of 10 ml. of water the 2-heptadecyl-2-imidazoline was added in 0.2 ml. of acetone and the uptake experiment started. Quantities of Tergitol 7 used were 12.5 and 25.0 mg. for 10- and 90-mg. lots of spores, respectively.

posure to the imidazoline was carried out as indicated in Table IX. Controls, not treated with Tergitol 7, were subjected to the same washing procedure. The spores which had been exposed to Tergitol 7 only took up about one-third to one-fifth as much of the imidazoline as the control spores during the first minute. After about 14 hours the Tergitol-treated spores still had taken out of solution only half of that taken out by the control spores. Reduction of the imidazoline content was in fact greater in the control tube, containing no spores, than with the spores which had been treated with the Tergitol.

In order to determine whether the uptake of silver was similarly affected 5.02 micrograms of silver were added to each of the tubes used in the above test and the experiment continued for another 156 minutes. During this period there was opportunity, therefore, for any unabsorbed imidazoline as well as for the silver to be taken up. Samples were taken at intervals of 1, 6, and 156 minutes and both the silver and the imidazoline determined. The results given in Table X show no interference in the uptake of silver that can be ascribed to the Tergitol 7. Changes in the imidazoline content of the supernatants were minor.

TABLE X

UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE AND SILVER FROM SOLUTIONS BY SPORES OF *NEUROSPORA SITOPHILA* PREVIOUSLY TREATED WITH THE IMIDAZOLINE

Spore wt., mg.	Tergitol 7 used	Imidazoline taken up previously, micrograms	Toxicant present in test, micrograms		Uptake after time intervals in minutes					
					1		6		156	
			Imidazoline	Ag	Im	Ag	Im	Ag	Im	Ag
10	No	22.6	4.16	5.02	-0.69	3.02	-0.11	3.11	-0.82	3.87
10	Yes	10.4	12.17	5.02	-1.01	2.69	-1.24	3.23	-0.46	4.02
30	No	26.7	2.47	5.02	-0.82	2.58	-0.65	2.93	-1.47	3.00
90	No	27.1	2.44	5.02	-0.25	3.34	0.15	3.83	-0.15	4.28
90	Yes	12.4	11.00	5.02	-0.88	1.59	0.26	3.02	3.56	4.34

In another test the spores were again pretreated as in the experiment summarized in Table IX but only 2.5 mg. of Tergitol 7 were used in each instance. Lots of 20 mg. each of spores of *Neurospora sitophila* were treated and in addition to 2-heptadecyl-2-imidazoline and silver, cerium was also included in the experiment. Even with this smaller quantity of Tergitol 7, the uptake of the imidazoline was markedly decreased and remained lower than in the control even after 22 hours (Table XI). The uptake of silver and cerium was unaffected.

When the quantity of Tergitol 7 was reduced to 50 micrograms but was allowed to remain in the tubes during the tests, results as shown in Table XII were obtained. The amount of the imidazoline taken up in five minutes was reduced to about half of that of the control but after 66 hours differences were no longer evident. Again there was no effect on silver uptake

TABLE XI

EFFECT OF TERGITOL 7 ON THE UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE, SILVER, AND CERIUM BY SPORES OF *NEUROSPORA SITOPHILA*

Spore wt., mg.	Toxicant	Quantity, micrograms	Tergitol 7 used*	Cumulative uptake in micrograms after various time intervals in minutes		
				2	12	1332
20	Imidazoline	32.2	Yes	0	7.4	17.8
20	Imidazoline	32.2	No	13.2	18.8	23.7
20	Imidazoline	32.2	Yes	1.5	5.2	14.7
20	Imidazoline	32.2	No	17.2	20.2	25.0
0	Imidazoline	32.2	Yes	5.3	8.0	13.3
0	Imidazoline	32.2	No	2.3	5.4	9.4
20	Silver	10.4	Yes	4.7	8.5	9.3
20	Silver	10.4	No	6.4	8.4	8.3
20	Cerium	100	Yes	16.7	12.2	22.0
20	Cerium	100	No	12.2	13.4	13.5

* Spores pretreated with Tergitol 7 as indicated in footnote to Table IX but only 2.5 mg. of Tergitol 7 used in each test.

TABLE XII

EFFECT OF TERGITOL 7 ON THE UPTAKE OF 2-HEPTADECYL-2-IMIDAZOLINE AND SILVER BY SPORES OF *NEUROSPORA SITOPHILA*

Spore wt., mg.	Toxicant	Quantity, micrograms	Tergitol 7, micrograms	Cumulative uptake in micrograms after various time intervals in minutes	
				5	3960
20	Imidazoline	32.2	50	9.3	23.2
20	Imidazoline	32.2	0	17.7	24.4
0	Imidazoline	32.2	50	3.8	17.4
0	Imidazoline	32.2	0	8.1	17.4
20	Silver	10.4	50	8.5	8.5
20	Silver	10.4	0	8.4	8.2
0	Silver	10.4	50	1.3	1.3
0	Silver	10.4	0	0.8	2.2

TREATMENTS WITH $Ce_2(SO_4)_3$ CONTAINING BOTH LABELED Ce^{+++} AND LABELED SO_4^{--}

Since $Ce_2(SO_4)_3$ ionizes readily one would expect the uptake of Ce^{+++} and SO_4^{--} ions to be independent of each other. With the availability of Ce^{144} and S^{35} labeled sulfate it was easily possible to check this point. A number of such experiments were carried out and data from one of which are shown in Table XIII. In this test, lots of spores of *Monilinia fructicola* from 10 to 320 milligrams in weight were treated with 10 ml. of a solution of $Ce_2(SO_4)_3$ containing 100 micrograms of Ce^{+++} and the uptake of Ce^{+++} and SO_4^{--} was determined at intervals of 1, 6, and 61 minutes. The data show removal of progressively larger amounts of cerium as the number of spores used increased. There was some removal of sulfate in each lot but

TABLE XIII

UPTAKE OF Ce^{+++} AND SO_4^{--} BY SPORES OF *MONILINIA FRUCTICOLA* FROM 10 ML. OF SOLUTIONS OF $Ce_2(SO_4)_3$ CONTAINING 10 P.P.M. OF CERIUM

Spore wt., mg.	Ce ⁺⁺⁺ and SO ₄ ⁻⁻⁻ taken up in micrograms after various time intervals in minutes						Ce ⁺⁺⁺ content after 61 minutes, p.p.m. of spore wt.*
	1.0		6.0		61.0		
	Ce ⁺⁺⁺	SO ₄ ⁻⁻⁻	Ce ⁺⁺⁺	SO ₄ ⁻⁻⁻	Ce ⁺⁺⁺	SO ₄ ⁻⁻⁻	
10	13.2	12.9	15.5	15.1	28.0	19.9	1861
20	16.9	23.8	36.4	23.3	34.3	22.5	1243
40	33.6	21.7	27.8	13.9	42.3	27.1	813
80	43.1	16.7	51.0	11.9	55.3	15.9	573
160	59.3	20.1	68.1	26.6	64.1	21.7	342
320	69.5	26.3	84.2	29.2	88.5	19.6	247
0	10.3	13.4	11.3	19.1	9.4	20.8	

* Corrected for loss of cerium in water control.

the quantities were about the same as those removed in the blank containing no spores. It is clear that there was a rapid and extensive uptake of Ce^{+++} while SO_4^{--} remained essentially in the external solution.

EXTRACTION OF TOXICANTS FROM TREATED SPORES

Several experiments were carried out on the removal of toxicants from treated spores. The results already reported in the present paper show clearly that resuspension in distilled water or further treatment with other fungicidal materials has little effect on releasing materials which have been taken up. Results of various extraction procedures in one experiment are shown in Table XIV. The imidazoline can be removed by treatment with absolute alcohol but this has little effect in removing cerium or silver either in single or mixed treatments. Ammonium hydroxide extracts some of the

TABLE XIV
EXTRACTION OF TOXICANTS FROM SPORES OF *NEUROSPORA SITOPHILA*
(10-MG. LOTS) WITH ABSOLUTE ETHANOL AND NH_4OH SOLUTIONS

Toxicant	Micro-grams	Percentage removed by successive extractions						
		2-Ml. portions of ethanol				2 Ml. H_2O	2-Ml. portions of NH_4OH soln.	
		1	2	3	4		1	2
Imidazoline	22.54	75	21	3.4	0.4	0.1	0.2	0.1
Silver	3.65	0.8	0.3	0.3	0.3	1.9	10.1	7.1
Cerium	7.2	7.1	2.5	2.5	2.1	27	25	11
Imidazoline	20.94	81	9.9	2.3	—	0.2	0.3	0.2
Silver	3.91	0	0.08	0.03	—	0.0	3.3	2.2
Silver	2.38	5.5	0.8	2.1	2.1	2.1	2.6	2.1
Cerium	32.38	0.10	1.4	0.3	0.9	4.1	13.8	6.1
Imidazoline	25.4	93	12	3.3	3.4	0.3	1.9	—
Silver	4.4	0	0.5	0.2	0.9	1.8	0.5	—
Cerium	36.8	4.4	1.2	0.4	1.2	2.0	18	—

silver and cerium. Presumably with silver this represents the element tied up as silver chloride. Continued extraction with ammonium hydroxide, not shown in the table, fails, however, to remove all or most of the silver and it is necessary to use nitric acid to accomplish this. Treatment with nitric acid also releases most of the cerium taken up. These results indicate that strong affinities exist between the cells and the toxicants.

DISCUSSION

Investigation of the effect of consecutive and simultaneous treatments with 2-heptadecyl-2-imidazoline, silver, and cerium seemed to be in order

especially because of the large quantities on a spore weight basis required to inhibit spore germination. With an ED₅₀ of from 5,000 to almost 10,000 p.p.m. one would hardly expect the imidazoline to be selective as to receptor sites. Since the imidazoline is surface active the question naturally arises as to whether these large quantities primarily cover the spore surfaces and act by sheer mass rather than because of direct interference with some essential metabolic process. If these quantities of toxicant are merely physically deposited on the cell surface they might impede, if only mechanically, the uptake of other toxicants which involve more specific receptor sites. Mere deposition on the surface of quantities equal to 1 per cent or more of the fresh weight of the spores would necessitate a thick layer in terms of molecules even if the spore surfaces are not smooth. With silver and cerium there might be a more direct competition for receptor sites.

In the light of the above considerations it would seem probable that previous treatment with silver or cerium would not affect subsequent uptake of the imidazoline but treatment with the imidazoline might have an influence on later uptake of silver or cerium. If silver and cerium have any receptor sites in common, affinity for one element would most likely be greater than for the other and thus a subsequent treatment with the element with the greater affinity should result in the release of some of the element previously taken up. The results of the experiments reported in this paper indicate a complete lack of influence of one of these toxicants on the uptake or release of the others, either in consecutive or simultaneous treatments. It would thus seem that the uptake of each of these toxicants is concerned with mechanisms which are different enough from each other so that there is no interference in the type of treatments used in these investigations. With each toxicant the mechanisms act rapidly since the greater part is usually taken out of solution in 30 seconds or less.

When similar studies are carried out on toxicants more closely related chemically than the imidazoline, silver, and cerium, indications of related or identical receptor sites will become evident. Preliminary experiments have shown that some of the other heavy metals interfere with the uptake and aid in the release of silver, and related rare earths affect the action of cerium. Determination of the degree and rate of exchange on the further addition of the same or related toxicant ions or compounds also gives information on relationships between toxicants as to receptor sites and reactions with compounds of the spores. Such investigations are now in progress and the results will be published in a subsequent paper.

Previous experiments (5), in which the relation between uptake and germination in the same lot of spores was studied for 2-heptadecyl-2-imidazoline, silver, and cerium, have shown that spores will take up much more toxicant than the quantity required to prevent germination. Similarly, in the studies on uptake with consecutive treatments, spores which had taken

up sufficient of one of the toxicants in question to impair seriously their germination capacity, were still able to take up large quantities of a second or even a second and third toxicant. This result raises the interesting question as to what degree various biochemical processes may still function for a time in fungus spores which are no longer able to germinate. In the studies on the formation of hydrogen sulfide from elemental sulfur by fungus spores (4), which probably depends upon a balance between at least two enzyme systems, it was concluded that the loss of ability to germinate coincided fairly closely with failure to continue hydrogen sulfide formation.

Evidence as to penetration or at least interaction between the toxicants and the treated spores can be obtained by determining the changes undergone by the toxicants as a result of treatment of spores and studying possible compounds formed between the toxicants and spore constituents. Preliminary experiments in which spores of *Neurospora sitophila* which had been treated with 2-heptadecyl-2-imidazoline were suspended in distilled water and the respiratory carbon dioxide examined have indicated that a small percentage of the C¹⁴ in the ring is recovered in the carbon dioxide given off. This indicates that at least this part of the imidazoline is not merely adsorbed on the surface but does become a part of some of the metabolic processes of the cells.

Previous investigations have shown that ED₅₀ values on a spore weight basis are quite high for the fungicides which have been studied. These values have ranged from 85 p.p.m. for silver and spores of *Venturia pyrina* to 250 to 540 for spores of other species. With cerium an ED₅₀ value of 4600 p.p.m. was found for spores of *Monilinia fruticola*, while values of 5000 to 9300 p.p.m. were found for spores of *Neurospora sitophila* and *Venturia pyrina* with 2-heptadecyl-2-imidazoline (5). In studies with sulfur (4), dosage as determined by the quantities of hydrogen sulfide which spores produced before germination capacity was seriously impaired, was again very high, 10,000 p.p.m. or higher. The early work of Marsh (2) showed that high doses of copper were involved in treatments of spores of *Monilinia fruticola* to reduce germination. These high doses can be compared with the efficacy of toxicants against other organisms. Substances lethal to man and other animals (1) are active at much lower doses than those indicated for the fungicides as are the more effective insecticides (3) and herbicides. With larger organisms it is necessary only to kill or inactivate a limited number of cells, the destruction of which in turn may lead to the death of the whole organism. This may account in part for the lower doses on a weight basis. Experiments with *Proteus vulgaris* and radioactive cobalt by Neyland, Dunkel, and Schade (6) have shown that the quantities of cobalt taken up by the bacterium on a weight basis at growth inhibitory levels are of the same order of magnitude as in the studies with fungicides. Penicillin,

on the other hand, acting on staphylococci is very effective on a weight basis. The uptake has been studied with aid of S^{35} labeled material by Rowley *et al.* (7) who found that concentrations within the bacteria of the order of only several parts per million prevent growth. It should be possible, therefore, to find fungicides more active on a weight basis than those which have been studied so far.

SUMMARY

Spores of *Neurospora sitophila*, *Monilinia fructicola*, *Aspergillus niger*, *Glomerella cingulata*, and *Venturia pyrina* were exposed to various combinations of 2-heptadecyl-2-imidazoline, silver, and cerium both in simultaneous and consecutive treatments. The results have shown that fungus spores take up these toxicants at about the same rate and to about the same degree whether they are presented to them singly, simultaneously, or consecutively. Toxicants taken up are not released on resuspension in water or on further treatment with a second toxicant. These results indicate that receptor sites for the three toxicants in question are not the same.

Pretreatment or simultaneous treatment with Tergitol 7 was found to interfere seriously with the uptake of 2-heptadecyl-2-imidazoline but was without effect on the uptake of silver or cerium.

When fungus spores were exposed to solutions of $Ce_2(SO_4)_3$ containing both labeled Ce^{+++} and SO_4^{--} , Ce^{+++} but little or no SO_4^{--} was taken up by the spores.

Drastic procedures were required to remove the toxicants from the spores even when they had only been in brief contact with the fungicidal solutions. The 2-heptadecyl-2-imidazoline could be extracted with ethanol. Solutions of ammonium hydroxide removed some cerium and silver from treated spores but the greater part was not extracted unless nitric acid was used.

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FACTORS ASSOCIATED WITH THE FUNGITOXICITY OF FERBAM AND NABAM¹

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Organic sulfur fungicides, such as ferric dimethyldithiocarbamate (ferbam) and disodium ethylenebisdithiocarbamate (nabam) have been in commercial use for some time and there has been considerable speculation as to their mode of action. These compounds exhibit a degree of instability and their effectiveness has often been attributed to known or probable decomposition products. The toxic vapor given off by aqueous solutions of nabam has been the subject of a number of papers (1, 4, 8, 18).

With the aid of S³⁵ labeled ferric dimethyldithiocarbamate² and by conventional chemical methods, studies have been carried out on the nature and effectiveness of some of these products and on factors influencing their formation. The chief volatile product from both ferbam and nabam, quantitatively, is carbon disulfide. The carbon disulfide from labeled ferbam has been shown to enter leaves near treated leaves on the same or adjacent plants and the sulfur from the carbon disulfide appears in the various sulfur fractions. Studies on the toxicity of carbon disulfide to fungus spores indicate that it is not sufficiently toxic to account for the action of these fungicides. The vapor from aqueous solutions of nabam is much more toxic than equivalent quantities of carbon disulfide or ethylenediamine, and a still unknown constituent must be involved. Ferbam reacts with sulfhydryl compounds, such as glutathione and cysteine, and, in fact, the toxic action to fungus spores can be at least partially reversed by these compounds. The decomposition of nabam is increased by oxidized glutathione. These reactions with essential cell constituents may play an important role in their toxicity to fungus spores.

MATERIALS AND METHODS

Chemicals

The ferric dimethyldithiocarbamate (ferbam) used was specially purified material supplied by E. I. du Pont de Nemours & Co., Inc. Sulfur labeled ferric dimethyldithiocarbamate was obtained from Tracerlab Inc., Boston, Mass. Bis(dimethylthiocarbamyl)disulfide (tetramethylthiuram disulfide, thiram), bis(diethylthiocarbamyl)disulfide (tetraethylthiuram disulfide, TETD), and disodium ethylenebisdithiocarbamate (nabam) were

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purified by recrystallization of commercial materials. Zinc dimethyldithiocarbamate (ziram) and zinc ethylenebisdithiocarbamate (zineb) were prepared by precipitation of their corresponding sodium salts with zinc acetate. Ethylene diisothiocyanate and hexamethylene diisothiocyanate were prepared according to the methods of Yakubovich and Klimova (21) and Klöpping (7). All other chemicals were reagent grade unless otherwise noted.

Plant Materials

Green plants used were tomato (*Lycopersicon esculentum* Mill. var. Bonny Best), bean (*Phaseolus vulgaris* L.), and cotton (*Gossypium hirsutum* L. var. Deltapine). Fungal materials used were conidia of *Monilinia fructicola* (Wint.) Honey, *Neurospora sitophila* (Mont.) Shear & Dodge, *Aspergillus niger* van Tiegh, *Rhizopus nigricans* Ehr., *Alternaria oleracea* Milbraith, *Glomerella cingulata* (St.) Sp. & von S., and dried yeast cells (*Saccharomyces cerevisiae* Hansen). Detailed methods for culture, harvest, and germination of fungus spores have been presented in a previous paper (14).

Uptake of S^{35} by Green Plants from Labeled Ferbam

The uptake by plants of S^{35} from ferbam was studied under conditions in which the ferbam, partly in solution and partly in suspension, was added to the root system. In other tests solid ferbam was placed on leaves and the S^{35} content of portions of the plant not in direct contact with the ferbam determined. Leaves were also exposed to vapor from dry ferbam. The uptake of radioactive carbon disulfide, obtained by acid treatment of labeled ferbam, was also studied.

When leaves were exposed to vapor from ferbam they were isolated from the rest of the plant by enclosing the petiole in a drilled and split rubber stopper of the appropriate size, then introducing both leaf and stopper into an 8-ounce extra widemouthed jar and sealing with lanolin. In some of the tests in which the ferbam was added to the roots, a similar sealing method was used to prevent vapor escaping from the immersed root system from entering the leaves directly.

When leaves were exposed to radioactive carbon disulfide they were sealed as described above with the stopper modified to include gas inlet and outlet tubes. The generated carbon disulfide was moved by means of nitrogen gas from the reaction vessel through a 2 per cent zinc acetate solution, then successively through the four jars enclosing leaves and finally into an absorber solution [Dickinson-Viles reagent (20)].

Plants were maintained at room temperature in an artificially lighted chamber during the test periods. They were sampled for analysis after exposure periods of two to eight days. Sulfate sulfur was determined by precipitation as barium sulfate with the addition of carrier sulfate. For the determination of total sulfur or nonsulfate sulfur the plant samples were di-

gested with nitric and perchloric acids to oxidize the sulfur compounds to sulfate. The radioactivity of the barium sulfate samples was determined with an end window type Geiger tube and conventional scaler. Appropriate corrections for self-absorption were made.

Determination of Rate of Decomposition of Dithiocarbamates

Decomposition of dithiocarbamates and related compounds with the production of carbon disulfide was studied with the use of the Dickinson-Viles reagent (20) which gives a color with small quantities of carbon disulfide. A suspension or solution of the compound to be studied was placed in a Drechsel gas-washing bottle and any gaseous products produced were removed by a stream of nitrogen, flowing at a rate of approximately 20 ml./min., and passed on through two more bottles, the first containing a 2 per cent solution of zinc acetate to remove any hydrogen sulfide produced and the second the Dickinson-Viles reagent. It was found that one tube of the reagent was sufficient to trap all the carbon disulfide formed. Colors were measured with a Klett-Summerson photoelectric colorimeter using a blue filter having an approximate spectral range of 400 to 465 millimicrons. Dickinson-Viles reagent was prepared by dissolving 60 mg. of cupric acetate $[\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}]$ in 10 ml. of distilled water, adding 20 ml. of triethanolamine, making up to 1 liter with 95 per cent ethanol and adding 1 ml. of diethylamine. Temperature was controlled at 29.5°C . by immersing the tubes in an electrically heated water bath.

Reversal of Ferbam Toxicity

To test the reversibility of toxicity of ferbam to spores of *Neurospora sitophila* by sulfhydryl compounds, spores were suspended in an aqueous solution of ferbam contained in a conical centrifuge tube. At the end of the planned exposure period the spore suspension was centrifuged and the ferbam solution decanted. The spores were resuspended in distilled water or a solution of a sulfhydryl compound, and allowed to stand for periods ranging from 10 to 60 minutes. At the end of the exposure period aliquots of the spore suspension were removed for determination of germinability.

Effect of Ferbam and Nabam on Urease

The effect of ferbam and nabam on urease activity was determined with the use of the colorimetric method of Van Slyke and Archibald (19). A saturated solution of ferbam was prepared by adding an acetone solution of ferbam to a buffer solution in a 1:100 ratio and filtering the resulting suspension. Nabam was used at concentrations of 2.5 and 2.5×10^{-1} mg./ml. The urease preparation was obtained from Nutritional Biochemicals Corp. Temperature was controlled at $26^\circ \pm 1^\circ \text{C}$. Color changes were observed after 4 and 24 hours' reaction time.

Toxic Properties of Vapors

Determinations of the toxicity to fungus spores in drops exposed to vapor being given off by solutions of fungitoxic chemicals were made with the use of a low form Stender dish with a capacity of 30 ml. Solutions to be tested were added in a volume of 2 ml. to the Stender dishes.

Drops of spore suspensions prepared by use of standard test techniques (14) were placed on 22-mm. cover slips which were supported above the solution to be tested by an 18 × 10-mm. micro slide ring (Fig. 1). The cover was sealed to the dish with a preparation described by Meloche and Frederick (12) as being unaffected by organic solvents. The material was prepared by mixing 60 g. anhydrous glycerol, 42 g. dextrin, and 10 g. D-mannitol and heating with constant stirring until a fairly clear viscous liquid was obtained. After holding at 21° C. for 20 to 24 hours the cover slips with the drops of spore suspensions were removed from the Stender dishes,

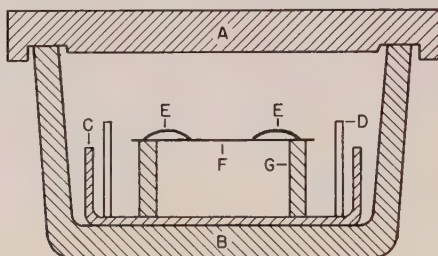


FIGURE 1. Section of apparatus used to study toxicity of vapor to fungus spores and the effect of absorbers on such toxicity. A, B, Stender dish and cover; C, glass container, 35 mm. in diameter and 10 mm. in height; D, segment of Soxhlet thimble used to give increased surface to absorbing solution; E, drops containing spores; F, cover slip; G, micro slide ring.

placed on glass slides, and the percentage germination was determined. The slide and cover slips were then transferred to a moist chamber and after an additional 18 to 24 hours the percentage germination was redetermined thus making possible an approximate differentiation between fungistatic and fungicidal effects.

The technique was modified as shown in Figure 1 to permit the addition of chemicals which could react with gaseous materials and thus prevent or reduce their effect on germination. A second glass container (C) having a diameter of 35 mm. and a height of approximately 10 mm. was inserted in the Stender dish. A 14-mm. segment of a 33-mm. diameter Soxhlet thimble (D) was inserted into the container and 2 ml. of a 5 per cent solution of the desired absorber was added. The micro slide ring (G) and the cover slip (F) were then placed in the container (C).

RESULTS

UPTAKE OF S^{35} FROM LABELED FERBAM

By fungus spores. When fungus spores distributed in 1-inch stainless steel planchets were exposed to the vapor from labeled ferbam in a closed container, considerable radioactivity was taken up by the spores. The ferbam was in the dry state except for any moisture that may have been obtained from the atmosphere of the closed containers. The data in Table I show that the planchets with the spores gave from 10 to 40 or more times as much activity as a paper disc placed in a planchet. This indicated clearly that either a sulfur-containing compound or sulfur vapor itself was being given off by the ferbam. Further tests reported below showed that the compound in question was carbon disulfide.

TABLE I

ABSORPTION OF RADIOACTIVITY BY FUNGUS SPORES EXPOSED TO THE VAPOR OF S^{35} LABELED FERBAM IN CLOSED CONTAINERS

Species	Counts per minute, after various exposure periods in hours				
	21	48	72	96	192
<i>Monilinia fruticola</i>	1342	2653	2759	2634	2700
<i>Rhizopus nigricans</i>	590	874	1045	1531	1940
<i>Aspergillus niger</i>	932	1758	2695	7380	9205
Paper disc	44	57	85	217	202

By the root system of green plants. The results of an experiment in which ferbam was added to the root systems of small tomato plants are summarized in Table II. The roots of plants six to seven inches tall were freed of soil and placed in 400 ml. of tap water in 500-ml. Erlenmeyer flasks. With two of the plants the stem above the root system was enclosed by a drilled

TABLE II

UPTAKE OF S^{35} FROM LABELED FERBAM BY THE ROOT SYSTEM OF TOMATO PLANTS

Root system in open or sealed containers	Fresh wt. of tops, g.	Ferbam added, micrograms	Per cent of activity recovered in tops after 5 days	
			As sulfate	As insol. organic S
Open	9.6	515	1.12	0.32
	7.5	206	1.25	0.36
	8.7	206	1.23	0.13
	9.3	206	1.31	0.29
	8.0	103	1.60	0.19
	7.5	103	1.82	0.59
Sealed	7.5	206	2.25	0.41
	13.5	103	3.97	0.59

and split rubber stopper and sealed with modeling clay thus preventing the escape of any vapor from the flasks. Quantities of ferbam varying from 103 to 515 micrograms were added to the water surrounding the root system of each plant. After five days the tops were harvested and the radioactivity in the sulfate sulfur and in the insoluble sulfur fraction determined. Radioactive sulfur was found in both fractions. The recovery of activity was about 1.4 to 4.6 per cent of that originally in the added ferbam. The presence of radioactive sulfate shows that the sulfur taken up from ferbam has entered into the sulfur metabolism of the plants. The higher recovery of radioactivity from ferbam with the plants in which the root system was sealed into the containers suggests that a gaseous product is involved. Accordingly, some experiments were carried out in which leaves of plants were exposed to the vapor given off by ferbam.

By leaves exposed to the vapor from ferbam. In these tests ferbam labeled with S^{35} was weighed into a widemouthed bottle and with the aid of a drilled and split rubber stopper individual leaves of small cotton, bean, or tomato plants were exposed to the vapors from ferbam while sealed off from the rest of the plant. After exposure periods of from six to eight days the exposed leaves and the unexposed portion of the plants were examined for radioactive sulfur. The data in Table III show that sulfur

TABLE III

PRESENCE OF S^{35} IN LEAVES EXPOSED TO VAPOR FROM FERBAM AND IN UNEXPOSED PORTIONS OF COTTON, BEAN, AND TOMATO PLANTS

Species	Ferbam present, mg.	Fresh wt., g.		Per cent of added S^{35} recovered	
		Exposed leaf	Unexposed tops	Exposed leaf	Unexposed tops
Cotton	23.6	0.40	2.7	0.33	0.009
	13.7	0.40	2.9	0.12	0.010
	7.4	0.34	2.8	0.27	0.0
Bean	24.2	—	4.5	—	0.0103*
	7.5	—	4.0	—	0.008*
Tomato	32	—	9.0	—	0.004*

* Values for the percentage of activity in the sulfate sulfur in these samples were 0.009, 0.006, and 0.002, respectively.

arising from the ferbam was absorbed and translocated. In those instances in which sulfate sulfur as well as insoluble organic sulfur were determined it is apparent that a substantial percentage of the S^{35} has been converted to the sulfate form. In another test a quantity of ferbam in aqueous suspension giving 31,328 counts per minute was placed on a leaflet of a young tomato plant (A). This plant was kept in a chamber together with another plant (B) which had received no application of ferbam. After

two days one leaflet from each plant was taken and the radioactivity in the total sulfur fraction determined. The sulfur from the leaflet from plant B gave 625 counts per minute and that from plant A gave 137 counts per minute. The leaflet from plant B was much nearer to the ferbam which had been applied to plant A and thus gave a much higher count even though it had no opportunity to receive sulfur other than through the vapor phase. This result further demonstrates the role of the vapor from ferbam in the movement of sulfur from applied ferbam into host tissue.

By leaves exposed to radioactive carbon disulfide. Other studies being carried out at the same time indicated that the volatile sulfur compound given off from ferbam was carbon disulfide. An experiment was set up in which individual leaves of bean plants were exposed to radioactive carbon disulfide (Table IV). It is apparent that carbon disulfide was absorbed by the bean tissue and the sulfur compounds formed were transported to other parts of the plant.

TABLE IV
UPTAKE OF RADIOACTIVE SULFUR BY BEAN LEAVES EXPOSED
TO LABELED CARBON DISULFIDE

Exposure period, hr.	Fresh wt., g.		Radioactivity recovered in total S, c./m.	
	Exposed leaf	Unexposed tops	Exposed leaf	Unexposed tops
1	0.50	2.5	147	16
21	0.70	2.0	841	16
21	0.50	3.0	969	41
21	0.85	2.0	667	31

DECOMPOSITION OF FERBAM AND NABAM

Identification of CS₂ as a Decomposition Product

The possibility that carbon disulfide is one of the toxicants involved in the fungicidal action of the dithiocarbamates was first suggested by Parker-Rhodes in 1943 (17). The formation of carbon disulfide in quantitative yields on the addition of strong acid is the basis of a method for the determination of the dithiocarbamates (5). Recent work by Cox, Sisler, and Spurr (4), Lopatecki and Newton (8), and Palmer, Greenlee, and Baldwin (16) which was carried out concurrently with the experiments reported in this paper and earlier (11), has shown that carbon disulfide is given off under conditions obtained when fungus spores are exposed to the fungicides.

In the present studies identification of carbon disulfide as a decomposition product of ferbam and nabam was made by use of a paper chromato-

graphic technique. The decomposition products were drawn through alcoholic-KOH solutions with a stream of nitrogen gas. Aliquots of these solutions and an alcoholic-KOH solution to which carbon disulfide had been added were placed on Whatman No. 1 filter paper and the chromatogram was developed with water-saturated butanol. The xanthate spots were made visible by using a dilute solution of copper sulfate as the chromogen. The R_f values for carbon disulfide and the volatile products from ferbam and nabam were identical.

Effect of Fungi on the Decomposition of Ferbam and Nabam

Since ferbam decomposes slowly to yield carbon disulfide it seemed of interest to determine whether fungal material such as yeast cells would have any effect on the rate of decomposition. The results of such a test are summarized in Table V. The quantity of carbon disulfide produced in the presence and absence of living yeast cells and yeast cells which had been killed by boiling in water for two minutes was determined. When 100 mg. of yeast cells were present the quantity of carbon disulfide produced was increased about 20 times compared with the control, and when heat-treated yeast was used the increase was only about 4-fold.

TABLE V
EFFECT OF YEAST, BOILED YEAST, AND WATER ON THE EVOLUTION
OF CARBON DISULFIDE BY FERBAM

Reactants	CS ₂ recovered in 24 hours	
	Mg.	Per cent of theory
Ferbam + yeast	3.5	9.1
Ferbam + H ₂ O	0.21	0.54
Ferbam, dry	0.05	0.13
Ferbam + yeast	3.95	10.3
Ferbam + boiled yeast	0.66	1.7
Ferbam + H ₂ O	0.18	0.46

The decomposition of nabam was also determined in the presence and absence of yeast cells and spores of *Neurospora sitophila*. About half as much carbon disulfide was given off with spores of *Neurospora sitophila* as with the yeast cells. Examination of the pH of suspensions of equal fresh weights of yeast cells and spores of *Neurospora sitophila* gave values of 4.9 with the yeast cells and 5.9 with the spores of *Neurospora sitophila*. It was also found that four times as much $N/100$ NaOH was required to bring the yeast cells to a pH of 7.0 as compared to the spores. Accordingly the effect of pH in the decomposition of both nabam and ferbam was studied.

Effect of pH on Rate of Decomposition

Studies on the effect of pH on carbon disulfide evolution showed that nabam is extremely sensitive to small changes in pH. The results of one such test are summarized in Table VI. Increase in acidity from pH 6.3 to 5.7 increased the carbon disulfide produced about 5-fold. Small changes in pH do not have nearly such a pronounced effect with ferbam.

TABLE VI
EFFECT OF pH ON THE RATE OF BREAKDOWN OF NABAM

Time, hours	Cumulative quantities of CS ₂ produced in micrograms at several pH values						
	5.7	5.8	5.9	6.0	6.1	6.2	6.3
1	11	8	5	3	2	2	2
4	47	39	34	27	19	13	10
6	76	63	47	35	24	18	14
22	194	189	149	117	80	69	56

In view of the marked effect of pH the role of yeast cells in the breakdown of nabam and ferbam was reexamined under controlled conditions as to pH. It is apparent (Table VII) that yeast has no effect on the decomposition of nabam other than that which can be ascribed to pH but the effect of yeast on the decomposition of ferbam is partially independent of the pH of the ambient solution. The relative pH independence of the yeast-ferbam system suggested a trial of some known constituents of yeast on the decomposition of ferbam.

*Effect of Sulfhydryl and Disulfide Compounds on the Decomposition of
Ferbam, Nabam, and Some Other Organic Sulfur Fungicides*

Tests on the effect of glutathione, which occurs in yeast in considerable quantities, and oxidized glutathione, on the breakdown of ferbam and

TABLE VII
EFFECT OF pH ON THE BREAKDOWN OF NABAM AND FERBAM BY YEAST

Treatment		Micrograms of CS ₂ recovered	
pH	With or without yeast	Nabam—16 hours	Ferbam—3 hours
5	—	350	0
	+	350	66
6	—	55	4
	+	55	73
7	—	2	0
	+	2	8
HOH	—	3	0
	+	152	22

nabam at pH 5, are summarized in Table VIII. Included in the tests on the effects on breakdown are also the disulfide cystine, and the fungicides ziram, zineb, thiram, and tetraethylthiuram disulfide (TETD). The results show that the decomposition of ferbam, thiram, and TETD is increased by glutathione but not by oxidized glutathione. The breakdown of nabam and zineb is hastened by the latter and not by the reduced form. Cystine, thiram, and TETD, which are also disulfides, also increase the breakdown of nabam. These reactions between the fungitoxic materials and biologically important sulfur compounds are probably important in the mechanisms involved in their toxic action.

TABLE VIII
INTERACTION OF SULFHYDRYL AND DISULFIDE COMPOUNDS WITH FUNGICIDES
TO PRODUCE CARBON DISULFIDE

Sulfide or disulfide added	Time of exposure, hr.	Cumulative quantities of CS ₂ (in micrograms) produced at pH 5 and 29.5° C.					
		Ferbam	Ziram	Nabam	Zineb	Thiram	TETD
Glutathione	1	79	20	84	0	124	38
	2	91	60	162	4	1984	80
	22	115	130	682	16	3794	481
Oxidized glutathione	1	2	9	166	0	0	0
	2	2	32	306	1	0	0
	22	12	130	956	85	0	0
Cystine	1	2	10	124	14	0	0
	2	2	20	208	17	0	0
	22	12	82	688	50	0	0
Thiram	1	2	8	810	80		
	2	3	16	994	110		
	22	21	77	1774	236		
Tetraethylthiuram disulfide (TETD)	1	2	10	380	43		
	2	3	18	562	66		
	22	20	62	1592	133		
Control	1	2	31	86	9	0	0
	2	2	54	159	13	0	0
	22	12	118	629	36	0	0

REVERSIBILITY OF FERBAM TOXICITY

In view of the fact that ferbam can react with glutathione, one might expect that the addition of glutathione would interfere with or remove the toxic effect of ferbam on fungus spores. A number of tests were carried out with spores of *Neurospora sitophila* in which treatment with ferbam was followed by exposure to glutathione or cysteine. There was some indication of reversal by these procedures but the effect was incomplete. Thus in one experiment in which spores were exposed to a saturated solution of ferbam for time periods varying from 0.25 to 10 minutes, and then

placed in water or in solutions of glutathione containing 1 mg. in 5 ml., the spores in the glutathione germinated from 6 to 10 per cent while those in water were completely inhibited. In another test in which 17.5 to 25.0 p.p.m. of ferbam were used subsequent exposure to cysteine gave an average of 75 per cent germination while spores not so treated gave only 10 per cent. In further experiments similar or less complete reversals were obtained.

An attempt was made to determine whether the toxicity of nabam could be reversed by oxidized glutathione using the technique described above. This procedure could not be carried out since the toxicity of nabam was no longer evident when spores were resuspended in a medium suitable for germination even after exposure to 100 p.p.m. of nabam for 24 hours. Such results were obtained with spores of both *Neurospora sitophila* and *Monilinia fructicola*, while the usual slide-germination test gave ED₅₀ values of 5 to 10 p.p.m.

EFFECT OF FERBAM AND NABAM ON UREASE ACTIVITY

The effect of ferbam and nabam on urease activity was studied using the method of Van Slyke and Archibald (19). The reactions were carried out at pH 6.7 and at $25^{\circ} \pm 1^{\circ}$ C. The concentration of urease was varied in the tests rather than that of the inhibitor because of the limited solubility of ferbam. Color changes resulting from the formation of ammonia were observed after 4 and 24 hours. The range of concentrations of urease was varied from 4×10^{-1} to 4×10^{-9} mg./ml.

Urease activity was partially inhibited at a concentration of 4×10^{-5} mg./ml. and completely at 4×10^{-7} mg./ml., by ferbam at a concentration of 2.5×10^{-2} mg./ml. Nabam had no effect on urease activity at concentrations as high as 2.5 mg./ml. in these tests. Carbon disulfide, dimethylamine and iron, the breakdown products of ferbam, had no effect on urease activity. These three breakdown products were used singly and in all possible combinations of two products at concentrations that would result from the complete breakdown of ferbam at a concentration of 2.5×10^{-2} mg./ml.

Since urease is known to have free sulfhydryl groups it might be postulated that the inhibition of urease by ferbam results from the demonstrated reactivity of ferbam with sulfhydryl groups and that the failure of nabam to inhibit urease is the result of its nonreactivity with sulfhydryl groups.

TOXICITY OF VOLATILE COMPOUNDS RELEASED BY NABAM

There has been considerable recent interest in the nature of the gaseous toxicant given off by nabam. Rich and Horsfall (18), in experiments in which the effect on the germination of spores of *Monilinia fructicola* and *Stemphylium sarcinaeforme* exposed in hanging drops was studied, have

shown that the toxicant is not hydrogen sulfide, sulfur dioxide, or ethylenethiourea. Cox, Sisler, and Spurr (4) conclude that the gaseous toxicants from nabam solutions are carbon disulfide and ethylenediamine.

Experiments in which the vapor emanating from solutions of nabam and of carbon disulfide containing equal absolute quantities of the toxicants was tested against spores of *Monilinia fructicola* and *Neurospora sitophila* are summarized in Table IX. The data represent the averages obtained in two separate experiments. In the fungistatic tests the vapor from nabam was about ten times as toxic as that from an equal quantity of carbon disulfide. In the tests designed to determine fungicidal effectiveness the vapor from nabam was from 60 to 100 times as toxic as that from

TABLE IX
TOXICITY OF VAPOR FROM NABAM AND CS₂ AS INDICATED BY THE
PERCENTAGE GERMINATION OBTAINED IN FUNGISTATIC (S)
AND FUNGICIDAL (C) TESTS

Concn., p.p.m., (2 ml.)	<i>Monilinia fructicola</i>				<i>Neurospora sitophila</i>			
	Nabam		CS ₂		Nabam		CS ₂	
	S	C	S	C	S	C	S	C
Control	97	97	97	97	98	98	98	98
3.2	96	96	96	96	99	99	98	98
10	57	47	95	95	79	80	98	98
32	35	29	94	95	17	18	98	98
100	16	12	83	95	0	0	69	98
320	0	0	8	69	0	0	8	94
1,000	0	0	0	71	0	0	0	83
3,200	0	0	0	0	0	0	0	6
10,000					0	0	0	0
ED ₅₀	50	50	500	3000	20	20	150	2000

carbon disulfide. The vapor from nabam was entirely fungicidal in that there was no recovery of the spores in column C, unlike that of CS₂ in which there was considerable recovery. That is, much of the toxic effect of CS₂ vapor is fungistatic. These results indicate that carbon disulfide is not an important factor in the toxicity of the vapor from nabam.

In similar tests with the vapor of ethylenediamine the ED₅₀ for spores of *Monilinia fructicola* was found to be about 3000 p.p.m. and for spores of *Neurospora sitophila* >320 p.p.m. The low toxicity in these experiments is no doubt partly the result of the low vapor pressure of ethylenediamine. In other tests in which the fungicidal effectiveness of carbon disulfide and ethylenediamine in solution in closed containers was determined for spores of *Neurospora sitophila*, *Alternaria oleracea*, *Monilinia fructicola*, and *Glomerella cingulata*, the ethylenediamine was from 10 to 180 times as toxic as carbon disulfide.

It was thought that by using possible absorbers in the vapor tests as

described under Materials and Methods, some information might be obtained on the chemical nature of the toxic vapor from nabam. For comparison CS_2 was also run as well as hexamethylene diisothiocyanate. It would have been preferable to use ethylene diisothiocyanate since it has been postulated as a possible decomposition product of nabam (7). However, preparations of ethylene diisothiocyanate have been unstable in our hands and could not be used for experiments continuing over a number of days; therefore, the hexamethylene analogue was chosen to represent this type of compound.

The tests were run twice against the spores of *Monilinia fructicola* and *Neurospora sitophila*. The three toxicants were studied at 4 to 7 concentrations each differing by a ratio of $\sqrt{10}$. From these results the ED₅₀ values were obtained. Finally the ratio of the ED₅₀ value in the presence of the absorber in comparison with the ED₅₀ value without the absorber was obtained and is given in Table X. A high value indicates that the toxicity of the vapor was greatly reduced by the absorber. It will be seen that all the absorbers markedly reduced the toxicity of nabam except zinc acetate which was without appreciable effect. With carbon disulfide, particularly in the fungicidal tests, the absorbers were essentially ineffective. This is further evidence that the toxic factor in the vapor of nabam is not carbon disulfide. The lack of effectiveness of the zinc acetate absorber on nabam also indicates that the toxic vapor is not hydrogen sulfide. Since only potassium hydroxide and silver nitrate were effective against

TABLE X

RELATIVE EFFICIENCY OF ABSORBERS IN REMOVING THE TOXIC FACTOR FROM THE VAPOR OF NABAM, CS_2 , AND HEXAMETHYLENE DIISOTHIOCYANATE (HMDIT), AS SHOWN BY THE INCREASE IN RATIO OF ED₅₀ VALUES OF FUNGICIDE WITH ABSORBER TO ED₅₀ VALUES WITHOUT ABSORBER IN FUNGISTATIC (S) AND FUNGICIDAL (C) TESTS

Absorber	Nabam		CS_2		HMDIT	
	S	C	S	C	S	C
A. <i>Monilinia fructicola</i>						
Potassium hydroxide	10	10	.5	.7	>30	>30
Potassium permanganate	10	5	.5	1	3	3
Silver nitrate	>30	>15	1	.3	>30	>15
Sodium arsenite	30	15	3	>3	2	3
Zinc acetate	2	2	.5	1	1	2
B. <i>Neurospora sitophila</i>						
Potassium hydroxide	>2000	>2000	14	2	800	>1000
Potassium permanganate	40	40	1	1	1	3
Silver nitrate	>60	>60	1	3	>30	>30
Sodium arsenite	30	30	1	>2	4	8
Zinc acetate	4	4	14	>2	3	4

hexamethylene diisothiocyanate it appears that the effective compound released by nabam may not be ethylene diisothiocyanate.

DISCUSSION

The instability of the dithiocarbamates and the probable importance of decomposition products in their action as fungicides have been the subject of much investigation. Parker-Rhodes (17) first suggested that carbon disulfide might be involved in the fungicidal action of the dithiocarbamates although he found, as have other workers since then, that neither carbon disulfide nor the alkylamine which would be liberated simultaneously, has a high level of toxicity. Barratt and Horsfall (1), working with disodium ethylenebisdithiocarbamate (nabam), stressed the formation of hydrogen sulfide in the decomposition and its importance as a toxicant to fungus spores. Later Rich and Horsfall (18) showed that the toxicity of vapor from aqueous solutions of nabam could not be ascribed to hydrogen sulfide but that it must be due to a still unidentified constituent. Cox, Sisler, and Spurr (4) also studied the toxicity of the vapor from nabam and concluded it could be attributed to carbon disulfide and ethylenediamine. In order for carbon disulfide to account for the toxic action of the vapor of nabam it must be effective at concentrations equivalent to those that could be attained from the decomposition of the nabam. In the data reported by Cox *et al.*, the lower limit of effectiveness of the carbon disulfide was not determined. The quantity tested was many times that which could have been formed if all the nabam in the tests had decomposed completely to form the maximum quantity of carbon disulfide possible. If one ascribes the effectiveness of the vapor from nabam to a combination of carbon disulfide and ethylenediamine then one is faced with the dilemma that ethylenediamine and carbon disulfide react to form a dithiocarbamate.

Concomitant with the interest in the decomposition of the dithiocarbamates in connection with their fungicidal effectiveness, their decomposition has been studied in relation to analytical procedures used for their determination. Dickinson (5) was able to get quantitative recovery of the theoretical quantity of carbon disulfide from ferbam by treatment with strong acid. Therefore, under these conditions no hydrogen sulfide was formed. More recently Clarke, Baum, Stanley, and Hester (3) and Lowen (9) have published papers concerned with decomposition of the dithiocarbamates to form carbon disulfide. The former authors outline two modes of decomposition for nabam, one resulting in the formation of 2 moles of carbon disulfide from 1 mole of nabam, and in the other 1 mole each of hydrogen sulfide and carbon disulfide are formed. The former reaction is stated to be quantitative at 100° C. in dilute acid; the latter is stated to be less well understood and to occur slowly at lower

temperatures. Lowen, in studying the decomposition of nabam, found varying quantities of hydrogen sulfide and carbon disulfide to be produced, depending upon the conditions, all involving strong acid. Usually much less hydrogen sulfide than carbon disulfide was produced. On aerating large quantities of solutions of nabam for five days Ludwig and Thorn (10) noted an odor of hydrogen sulfide for only about the first hour.

In the tests with ferbam covered in this paper, which were carried out at pH values in the physiological range, no hydrogen sulfide was detected as a product of its decomposition. Although solutions of nabam smell of hydrogen sulfide, quantitative determinations of the amounts of both hydrogen sulfide and carbon disulfide recovered on passing nitrogen through the solutions under various conditions have always yielded from about 2.4 to 6.3 times as much carbon disulfide as hydrogen sulfide. Lopatecki and Newton (8) also report no hydrogen sulfide as a product of the decomposition of the metallic salts of dimethyldithiocarbamic acid but found about a 1.2 to 1 ratio of hydrogen sulfide to carbon disulfide in the gases given off by nabam at a pH of 6.0. These results seem to show clearly that hydrogen sulfide cannot play a role in the toxicity of the salts of dimethyldithiocarbamic acid. With nabam the situation is more complicated. In addition to carbon disulfide and hydrogen sulfide an unidentified volatile constituent is involved. Furthermore, Ludwig and Thorn (10) found that nabam solutions on aeration contain ethylenethiuram monosulfide as well as ethylenethiourea and other unidentified products. They report an ED₅₀ value of 1.5 p.p.m. for ethylenethiuram monosulfide against spores of *Monilinia fructicola*.

The fact that the fungicidal organic sulfur compounds react with glutathione, or oxidized glutathione, and with sulfhydryl-dependent enzymes such as urease would seem to be an important factor in their toxic action on spores. The chemistry of some of the reactions involved remains obscure. With tetramethylthiuram disulfide and glutathione the reaction presumably is a reduction of the disulfide to the corresponding dithiocarbamic acid which, in turn, is hydrolyzed to carbon disulfide. Such a reaction has been postulated by Johnston (6) to explain the reversal by glutathione of the blocking by tetraethylthiuram disulfide of liver aldehyde dehydrogenase. Tetramethyl-, tetrapropyl-, and dipyridylthiuram disulfide were also found to react with glutathione.

Since ferbam decomposes so readily to give off CS₂ it was not possible to tell in these experiments whether any ferbam as such was taken into host plants. While it was established that radioactive sulfur entered the plants in the form of carbon disulfide vapor, the possibility of some sulfur entering directly by absorption through the treated leaves was not necessarily excluded. The question as to whether ferbam as such enters the host

plant is difficult to answer because of the readiness with which the molecule breaks down into carbon disulfide and dimethylamine. Labeling atoms in the molecule other than the sulfur would not furnish an answer either. In view of the instability of ferbam at the pH of plant sap it would seem unlikely that small amounts of ferbam would remain intact for any length of time in plants. Similarly Lopatecki and Newton (8) suggest that there is little likelihood that nabam would be translocated unchanged within the plant.

The readiness with which carbon disulfide seems to give up its sulfur in plants is of interest. The plant seems to be able to obtain its sulfur needs from a great variety of sulfur compounds (2), such as hydrogen sulfide, elemental sulfur, sulfur dioxide, cystine, and DL-methionine (13), in addition to sulfate, the conventional source.

SUMMARY

Ferric dimethyldithiocarbamate (ferbam) labeled with S^{35} was found to give off a volatile sulfur compound which was taken up readily by fungus spores and green plants. Labeled carbon disulfide obtained from the ferbam by treatment with strong acid was also taken up by green plants. Using paper chromatograms, carbon disulfide was identified as the sulfur-containing volatile decomposition product of ferbam and disodium ethylenedisithiocarbamate (nabam). The sulfur absorbed was found in the sulfate and organic sulfur fractions, which shows that carbon disulfide can be metabolized by green plants.

The decomposition of ferbam and nabam to yield carbon disulfide was considerably increased as pH values decreased below 7.0. The decomposition to yield carbon disulfide was also affected by sulfhydryl compounds such as glutathione and cysteine with ferbam and by oxidized glutathione with nabam. These reactions with essential cell constituents probably are important in the toxic action of these fungicides.

Glutathione and cysteine partially reversed the toxic action of ferbam. Tests on reversal of the fungitoxicity of nabam could not be carried out since spores which had been exposed to as much as 100 p.p.m. for 24 hours germinated on removal from the toxicant.

Ferbam was effective in inhibiting the sulfhydryl-dependent enzyme urease while nabam had little effect.

A method based on the use of a Stender dish is described by which it is possible to test the fungistatic and fungicidal effectiveness of volatile toxicants and to study the ability of various absorbing solutions to remove the toxicant. Results obtained indicate that neither carbon disulfide nor ethylenediamine are responsible for the toxicity of vapor from solutions of nabam. Preliminary tests also indicate that the volatile toxicant is probably not a diisothiocyanate.

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IMPROVED METHODS FOR REARING LARVAE OF *Aedes* *Aegypti* (L.) FOR USE IN INSECTICIDE BIOASSAY¹

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INTRODUCTION

In a previous publication (2) a method was described for the bioassay of organic insecticides based on their ability to inhibit the phototaxic response of larvae of *Aedes aegypti* (L.). The larvae were confined behind a porous barrier in a glass trough, and then released with simultaneous exposure to an intense light source. Those which failed to migrate beyond a predetermined point during a period of one minute were classed as inactivated. Using this technique the T₅₀, or time required to immobilize 50 per cent of the population, was estimated by making readings at various intervals after the introduction of the toxicant.

The photomigration technique is potentially useful for bioassay since T₅₀ values can be obtained in an hour or less at concentrations of 0.1 to 1.0 p.p.m. of many commercially important insecticides. The fact that the larvae are automatically segregated into *living* and *moribund* classifications insures that the test results will be unbiased and minimizes sampling errors through ability to assess objectively the responses of large numbers of larvae.

Early in this work it was realized that fluctuations in the vitality of the larvae would tend to nullify the advantages gained by the photomigration method unless better rearing conditions could be developed. An attempt was made to secure uniformity through careful regulation of temperature, rearing time, and nutrient composition, but without any basic changes in the procedure described by Hartzell and Storrs (4). Test results improved measurably, but larval reproducibility still was not good enough to permit comparisons of data obtained on different days. Furthermore, the larvae often floated on the surface of the test medium and behaved erratically in their response to toxicants. At such times satisfactory results could not be obtained even by direct comparison to standards.

In order to reduce variability, a number of factors which affect larval resistance were investigated using as a criterion their response in the photo-

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migration test at a standard concentration of insecticide. Age, instar, and environmental factors were regulated, and eventually a method was developed for rearing larvae with relatively constant resistance characteristics. This paper outlines the rearing conditions now in use in these laboratories. Some of the details have been published elsewhere (2, 3, 4), but a complete description is given in the following sections to provide a convenient reference source for details on rearing mosquito larvae for use in bioassay.

MATERIALS AND METHODS

TESTING TECHNIQUES

The resistance of the larvae was measured using purified samples of heptachlor [1 (or 3a), 4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene] and chlordan [1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene] as reference insecticides. Stock solutions were prepared in Carbitol [2-(2-ethoxyethoxy)ethanol] at concentrations of 10 p.p.m. For each test, 1 ml. of a stock solution was added with swirling to 99 ml. of a 1.0 p.p.m. aqueous solution of Pluronic F-68 containing 100 larvae of *Aedes aegypti*. The suspension was then transferred to the migration chamber previously described (2) and after the onset of paralysis, the percentage of larvae immobilized was determined at two- to three-minute intervals. T₅₀ values were then interpolated from time-inhibition curves.

Heptachlor at a concentration of 0.1 p.p.m. in the final dilution was used as a primary reference standard in assessing larval resistance. In experiments where higher or lower concentrations were desired, the concentration of the stock solution was adjusted so that the test suspension always contained 1 per cent of Carbitol.

CULTURE MEDIA AND EQUIPMENT

A solution containing 1 per cent dextrose and 0.15 per cent U.S.P. XIV salt mixture was used to stimulate hatching. The nutrient for rearing culture and test larvae was prepared by mixing 50 grams of brewer's yeast (U.S.P. XII, supplied by Nutritional Biochemicals Co.), 30 grams of blood albumin (Fischer Scientific Co.), and 20 grams of sucrose. The mixture was blended thoroughly and stored in a brown bottle with a tight lid. Each day 2.5 grams of the dry mixture were weighed out into a mortar and ground into a smooth paste with a little water. The paste was then diluted to 1 liter with distilled water and aliquots of this suspension were added to the flasks containing the larvae. Pluronic F-68 (a polyethylene-polypropylene glycol supplied by the Wyandotte Chemical Co.) was used as a conditioning agent in the culture and test solutions.

All glassware used for rearing and testing was washed successively

with acetone and tap water and boiled in 2 per cent trisodium phosphate for one hour. It was then soaked in sulfuric acid-dichromate solution for at least 10 minutes and rinsed 20 times in running tap water.

Adults and larvae were grown in a basement room regulated at $28^{\circ} \pm 1^{\circ}$ C. with an air conditioning unit. Culture larvae to replenish the adult colony were grown in a warm air cabinet regulated at $32^{\circ} \pm 1^{\circ}$ C. Larvae for the photomigration test were reared in a 12-liter flask immersed in an eight-cubic foot constant temperature bath regulated at $29.5^{\circ} \pm 0.5^{\circ}$ C. Resistance to insecticides was measured in a separate room maintained at 25° to 26° C.

The adult colonies were maintained in 30- \times 30- \times 30-inch wooden cages with three sides enclosed. The front contained a 23- \times 11-inch glass window, a 10- \times 13-inch screen, and a 10- \times 13-inch opening covered by a cheesecloth entry tube to provide for light, ventilation, and the transfer of materials.

MAINTENANCE OF ADULT COLONIES

Two adult colonies were established from the Orlando strain previously described (2). The population density and the average age were stabilized by the daily introduction of newly emerged imagos.

The adults were fed daily on a 1 per cent dextrose solution contained in a 125-ml. Erlenmeyer flask equipped with a cotton wick. Two guinea pigs held in place on wooden blocks were introduced into each cage for one hour daily to supply the blood meal required by the female mosquitoes. Sixteen guinea pigs were used in rotation. Each animal was clipped and introduced into a cage every fifth day.

Strips of No. 1 Whatman filter paper 1.5 inches wide and 4 inches long were moistened and attached to the sides of 250-ml. beakers. These were then filled with distilled water until the lower edges of the papers were immersed. One beaker was placed in each cage daily. The mosquitoes deposited their eggs on the filter paper near the surface of the water. The strips containing the eggs were collected at 24-hour intervals and washed free from debris. They were then stored on moist filter paper in a closed container for five days. After this period they were air dried for more permanent storage. The desiccated eggs were kept as a reserve supply in the event of failure of the adult colony.

LARVAE FOR PHOTOMIGRATION TESTS

Pyrex test tubes containing 10 ml. of dextrose-saline solution were heated in a hot water bath for 5 to 10 minutes to drive off dissolved oxygen. The tubes were cooled to 33° C. and filter paper strips containing moist five-day-old eggs were placed in them for 15 to 30 minutes depending upon the rapidity of the hatch. The unhatched eggs were then removed, and the

solutions containing the young larvae were diluted with water and transferred to a large Petri dish.

Two thousand larvae were counted out with the aid of a medicine dropper and a hand tally counter and transferred to a 12-liter flask containing 8 liters of a 10-p.p.m. solution of Pluronic F-68. The flask was then immersed in a water bath maintained at 29.5° C. and stoppered with a cotton plug.

After 18.5 hours, 250 ml. of freshly prepared nutrient were added to the flask. After the addition of the nutrient the larvae were incubated an additional 22 hours. At the end of this period they were examined to determine their stage of development. If most of them were second instar, they were removed from the nutrient by filtering through a 60-mesh sieve set in a shallow pan arranged so that it could overflow without allowing the larvae to become exposed to the air. They were then washed with *ca.* 2 liters of a solution containing 10 p.p.m. Pluronic F-68, counted out into groups of 100, and held in 150-ml. beakers until used. Ten ml. of wash solution were included in each beaker so the concentration of Pluronic F-68 would be 1.0 p.p.m. when diluted to prepare the test suspension. In cases where large numbers of first instar larvae were still present at 22 hours, the incubation period was prolonged an hour or two to permit further molting. The remaining first instars were then sorted out and rejected.

REARING OF CULTURE LARVAE

Larvae for the maintenance of the adult colonies were hatched as described above, and groups of 250 placed in each of six 2-liter cotton-stoppered Erlenmeyer flasks containing 1 liter of distilled water and 50 ml. of the concentrated nutrient suspension. The flasks were then incubated at 32° C. in a thermostatically controlled warm air cabinet. As the larvae grew, their need for nutrients increased and contaminants built up within the flasks; thus the following schedule was used for adding nutrients and changing the rearing medium.

First day. The larvae were hatched and placed in the rearing medium. *Second day.* Fifty ml. of nutrient solution were added to each flask. *Third day.* The larvae were separated from the culture medium on a 60-mesh screen and transferred to clean 2-liter Erlenmeyer flasks containing 1 liter of distilled water. One hundred ml. of concentrated nutrient suspension were then added to each flask, and the flasks returned to the incubator. *Fourth day.* One hundred ml. of concentrated nutrient suspension were added to each flask. *Fifth day.* Pupae usually appeared by the fifth day. The remaining larvae and the pupae were washed free of rearing medium on a 60-mesh screen and transferred to pint Mason jars in 400 ml. of distilled water. Twenty-five ml. of nutrient suspension were added to each

jar and several small corks provided to support the newly emerged adults. Cheesecloth was fastened securely over the top of each jar, and the jars returned to the incubator until the adults emerged. *Sixth, seventh, and eighth days.* Adults were released into the cages. The adults emerged over a three-day period and each cage was supplied daily by the larvae from three jars. Fresh culture larvae were started Mondays, Wednesdays, and Fridays, hence young adults were supplied to the colony on a continuous basis.

EXPERIMENTAL RESULTS AND DISCUSSION

AGE AND INSTAR OF TEST LARVAE

During investigations on methods for rearing larvae suitable for the photomigration test it was necessary to consider a number of diverse factors. Some of these are concerned with the age and instar of the larvae and others with environmental conditions such as control of temperature and nutrients. Although these are all interrelated in their effects on growth and resistance to toxicants, it is convenient to consider them separately and first examine the relationship of larval age and instar to resistance, under the rearing conditions finally developed.

In earlier work (2, 4) larvae were used after 44 hours' incubation in the nutrient solution at 29° C. It was assumed that they were third instars, although no particular attention was given to confirming this on a daily basis. When the problem of improving reproducibility arose it was first decided to investigate the possibility of using younger larvae to increase the sensitivity of the test and provide for a more convenient rearing schedule with a faster turnover of equipment. The most promising procedure appeared to be the use of newly hatched larvae since these would not require nutrients and many of the difficulties encountered in rearing could be avoided entirely. However, it was found that the response of freshly-hatched larvae to light is not fully developed and they do not migrate satisfactorily during the first four to six hours. Furthermore, their resistance to heptachlor at a concentration of 0.1 p.p.m. as measured by T₅₀ proved to be high and variable.

These preliminary observations were confirmed by measuring T₅₀ as a function of larval age and instar on a single population reared under the conditions described in the preceding section. The T₅₀ at 0.1 p.p.m. heptachlor was determined immediately after hatching and larvae were then withdrawn at intervals and tested until pupation. The data (Fig. 1) show that the initial resistance was very high and that it dropped rapidly during the first 12 hours. Following this, there was a gradual increase until the first molt when the resistance dropped suddenly. This is illustrated by time-inactivation curves obtained separately on first and second instar larvae segregated from the same batch during the molting period (Fig. 2). The T₅₀ on the first instar larvae was 41 minutes while the second instars,

which were the same age but had already cast their skins, reacted to the toxicant in 28 minutes. This is possibly caused by higher penetrability of the new cuticle. Since the larvae cast their skins over a period of time, the drop in resistance was not as abrupt when tests were made without segregating the larvae according to instar. The data (Fig. 1) show that after the first initial drop, resistance increased in a series of steps from the first to the fourth instar. During each stadium the T₅₀ increased slowly

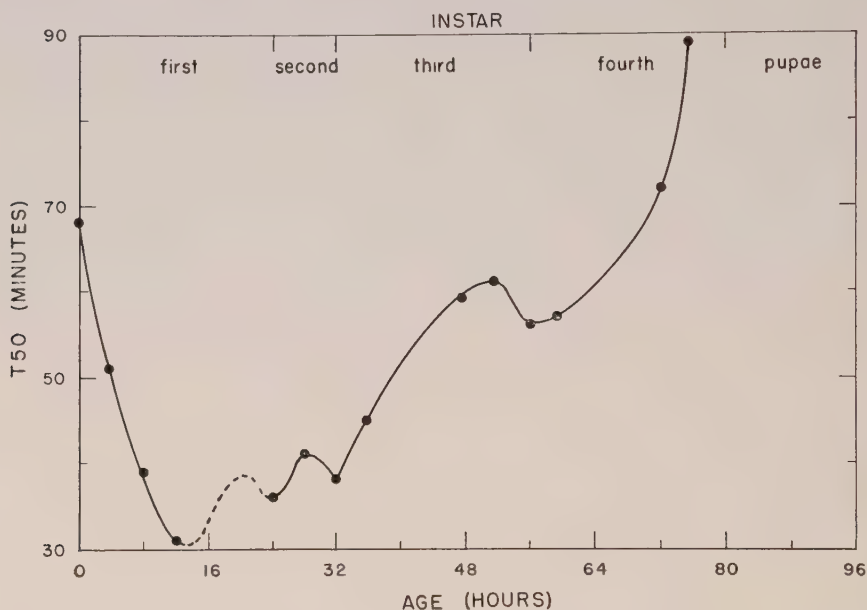


FIGURE 1. Change in the resistance of mosquito larvae with age and instar as measured by T₅₀ at a concentration of 0.1 p.p.m. heptachlor.

and at each molt dropped abruptly when the old skins were cast. The average level of resistance increased at each instar, and by the end of ~~72~~⁷² hours the resistance exceeded that obtained immediately after hatching.

The best time for testing is shortly after a molt, for then the resistance is low and the larvae can be maintained in distilled water for a considerable length of time without danger of their going over into the succeeding instar. However, molting should be complete or the earlier instar larvae should be segregated out, otherwise the tests will be made on mixed populations having distinctly different resistance characteristics. In such cases the time-resistance curves are highly skewed since a portion of the population succumbs rapidly and the rest may remain viable for a considerable length of time. Furthermore, larvae which are on the verge of molting will continue to do so even when removed from the nutrient, and

the characteristics of the population will change throughout the testing period. It is desirable therefore to adjust conditions so that molting will occur at a predetermined time, and, when necessary, segregate out the smaller larvae.

Under the rearing conditions used here, the third stadium lasted 24 hours and the second stadium about 8 hours. Even though third instar larvae are more stable, the second instars offer certain other advantages for insecticide bioassay. The T_{50} at 0.1 p.p.m. heptachlor is considerably

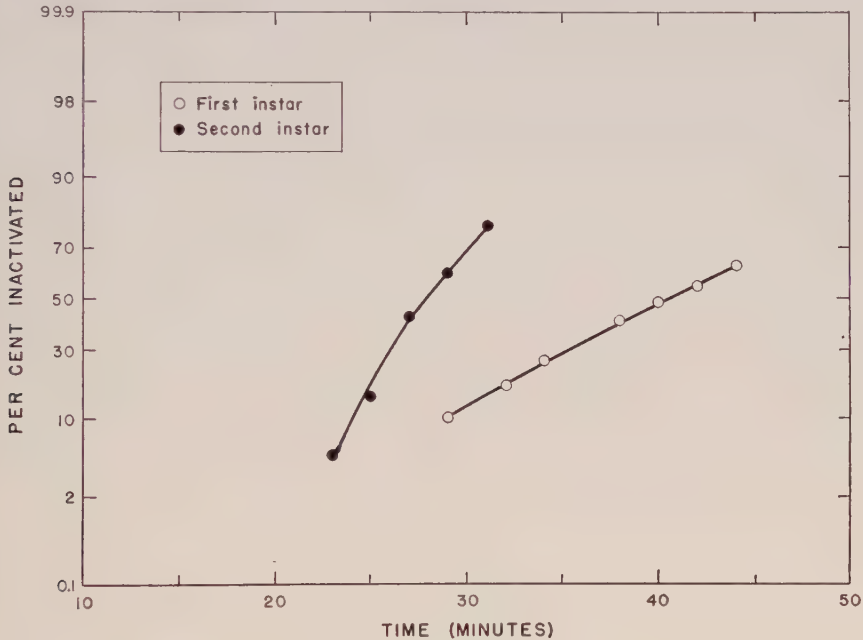


FIGURE 2. Comparative rates of inactivation of first and second instar 22-hour larvae in a solution containing 0.1 p.p.m. heptachlor.

lower at 22 hours than at 44 hours (Fig. 1), which accelerates testing and also increases sensitivity. This is particularly important for some of the slower acting chemicals which are difficult to detect at concentrations below 0.1 p.p.m. because of their high T_{50} values.

The use of second instar larvae also shortens the rearing schedule and lessens toxic effects due to spoilage of the nutrient. The most convenient rearing time for obtaining large batches of larvae suitable for a complete day's testing is 22 hours. This has been adopted for general use with minor variations to allow for differences in growth rates.

Even under the best environmental conditions, the yield of second instar larvae at 22 to 24 hours is variable. Occasionally first instars pre-

dominate, and it is necessary to sort out the seconds with a considerable loss in time and material. This situation was improved by hatching the larvae 18.5 hours in advance and maintaining them in distilled water until the introduction of the nutrient. Freshly-hatched larvae contain a reservoir of yolk and do not feed externally during the first few hours. Preconditioning them in distilled water gives them a start in development and apparently levels out individual differences enough to provide more uniform molting.

ADJUSTMENT OF ENVIRONMENTAL CONDITIONS

In the procedure used by Hartzell and Storrs (4) the larvae were fed on dog biscuit and reared in indefinite numbers in shallow enameled pans. In earlier work on the photomigration test (2) the importance of population density was established and the nutrient was changed to a mixture of yeast, blood albumin, and sucrose in the ratio of 5:1.5:1 at a total concentration of 0.65 g. per liter of culture medium. In this work the ratio was adjusted to 5:3:2 and the total concentration reduced to *ca.* 0.076 g./l., since the rearing time was to be shortened from 44 to 22 hours. The food mixture was prepared in advance and carefully homogenized with water in a mortar before adding it to the cultures. When added to the water as a dry powder it aggregated in clumps and the development of the larvae was delayed. Even with these changes, larvae reared in shallow pans varied in growth from pan to pan and with their position in the incubator. Measurements showed that pan temperatures were from 1° to 3° C. lower than the cabinet temperature owing to surface evaporation. The procedure was therefore altered to rearing the larvae in 2-liter cotton-stoppered Erlenmeyer flasks containing 1 liter of nutrient and 250 larvae per flask. This improved temperature control and resulted in better uniformity in growth and resistance. Subsequently the batch size was increased, and *ca.* 2000 larvae were reared in 8 liters of nutrient in a 12-liter round-bottomed flask immersed in a water bath maintained at $29.5^{\circ} \pm 0.5^{\circ}$ C. This eliminated interbatch variations entirely, and reduced the amount of labor required to produce larvae for the photomigration test. There has been no evidence so far that the smaller surface-volume ratio obtained in the flasks hinders development.

Aside from variations in resistance caused by irregular growth rate, the greatest source of error in the T₅₀ test was due to the fact that some larval populations floated on the surface of the test medium as if they contained occluded air bubbles or were covered by an oily film. Such larvae responded irregularly to toxicants and frequent inversions were obtained in T₅₀ values. Furthermore they were so difficult to handle in making transfers and counting that consecutive migration tests could not be made on schedule. The incidence of floating was reduced somewhat

if the larvae were kept immersed in water during the entire screening and washing procedure. However, the improvement was not sufficient to allow for satisfactory day-to-day testing.

A number of surface-active agents were examined to find a material that would reduce the interfacial tension between the larvae and the test medium. Vatsol OT, Triton X-100 and Pluronic F-68 at a concentration of 1.0 p.p.m. reduced floating without injury to the larvae. Triton X-100 and Vatsol OT inhibited the action of heptachlor, probably through solubilization in the micelles. Pluronic F-68 was noninhibitory at concentrations up to 100 p.p.m. Larvae remained viable as long as 24 hours in 1 to 20 per cent solutions but at this level the rate of insecticidal action was seriously reduced. In view of these findings, Pluronic F-68 was incorporated in the culture medium and in the water used for washing the larvae at a concentration of 10 p.p.m., and in the test suspension of insecticide at 1.0 p.p.m. This virtually eliminated floating and produced no adverse effects either on larval growth or on susceptibility to toxicants.

In addition to factors related to the rearing of larvae, the way in which eggs were treated prior to hatching also affected growth and viability. Desiccated eggs hatched over a period of several hours and mixed instars were usually obtained at the end of the rearing period. Since a continuous supply of eggs was available from the adult cultures they were used on a definite schedule to insure uniformity. Each day's hatch was transferred to moistened filter paper and maintained at 100 per cent humidity for five days to allow for maturation of the embryos. When placed in glucose-saline solution they hatched in 15 to 30 minutes, and at the end of the rearing period the larvae were more uniform than those obtained from dry eggs.

REPRODUCIBILITY OF PHOTOMIGRATION TESTS

Earlier work on the reproducibility of T₅₀ results on larvae withdrawn from the same batch indicated that there was no significant change in rate of inactivation during the first 1.5 hours after removal from the culture medium (2). Since it is desirable to rear large enough batches to provide larvae for use over an eight-hour day, this problem was reinvestigated using early second instar larvae to determine if it was necessary to correct T₅₀ values for time after removal from the nutrient.

Batches containing *ca.* 2000 larvae were washed free of nutrient and counted out into groups of 100 after rejection of the first instars. They were then tested at a single concentration of insecticide at various intervals throughout the day. Tests were made on chlordan at 0.50 p.p.m. and on heptachlor at concentrations of 0.01 to 0.20 p.p.m. on different days (Table I). Time after removal was taken as the difference between the time at which the larvae were washed free of nutrient and the time of 50 per cent

inhibition toward photomigration in order to compensate for widely differing inactivation periods. During the first eight hours after removal, resistance as measured by T_{50} increased linearly (Fig. 3). Thus the T_{50} at zero time (T_{50}^0) can be calculated from the relation:

$$T_{50}^0 = T_{50} - kt$$

where k is the slope of the line and t is the time after removal. For periods longer than eight hours this relationship breaks down since resistance tends to reach a maximum value.

Data obtained at various concentrations of chlordan and heptachlor were evaluated by the least squares method to obtain values for k and T_{50}^0 (Table I). The slope of the line varied between 0.36 and 1.33, and the

TABLE I
REGRESSION CONSTANTS FOR CHANGE IN T_{50} WITH TIME AFTER REMOVAL
OF LARVAE FROM CULTURE MEDIUM OBTAINED AT VARIOUS
CONCENTRATIONS OF CHLORDAN AND HEPTACHLOR

Insecticide	Concn., (p.p.m.)	T_{50}^0	k	s^*
Chlordan	0.50	32.1	0.65	2.25
Heptachlor	0.20	25.9	0.36	1.57
Heptachlor	0.10	28.2	1.19	2.02
Heptachlor	0.10	28.7	0.84	2.47
Heptachlor	0.10	28.8	0.81	1.52
Heptachlor	0.10	31.6	1.02	2.09
Heptachlor	0.06	35.2	0.80	1.81
Heptachlor	0.02	55.6	0.40	2.15
Heptachlor	0.01	106.8	1.33	6.63

* Standard error of estimate.

average weighted value was 0.73 minute per hour. Therefore all T_{50} results subsequently obtained were corrected back to zero time by subtracting 0.7 minute from the observed value for each hour after removal from the nutrient.

Values obtained for k on different batches of larvae show considerable variation. There is a tendency for k to increase as the T_{50} becomes very high. However, above 100 minutes the reproducibility of measurement is too low to give the correction much significance.

Once T_{50} values have been corrected for time after removal from the nutrient it is possible to obtain a reliable estimate of the error inherent in the test procedure. This is about 1.5 to 2.5 minutes in the 30-minute range. In view of this, it does not appear to be worthwhile to introduce more elaborate timing and transfer equipment until precision can be improved by reducing variations due to absorption effects, etc.

The correction of T_{50} values for time after removal from the nutrient is a worthwhile expedient, but it might be possible to obtain better results

by keeping the larvae overnight in distilled water until they reach constant resistance. Limited data indicate that the variation in T_{50} with time is negligible between 24 and 32 hours. However, some of the larvae molt and enter the third instar during this period and more confirmatory evidence is necessary. This would add an additional step to the procedure and

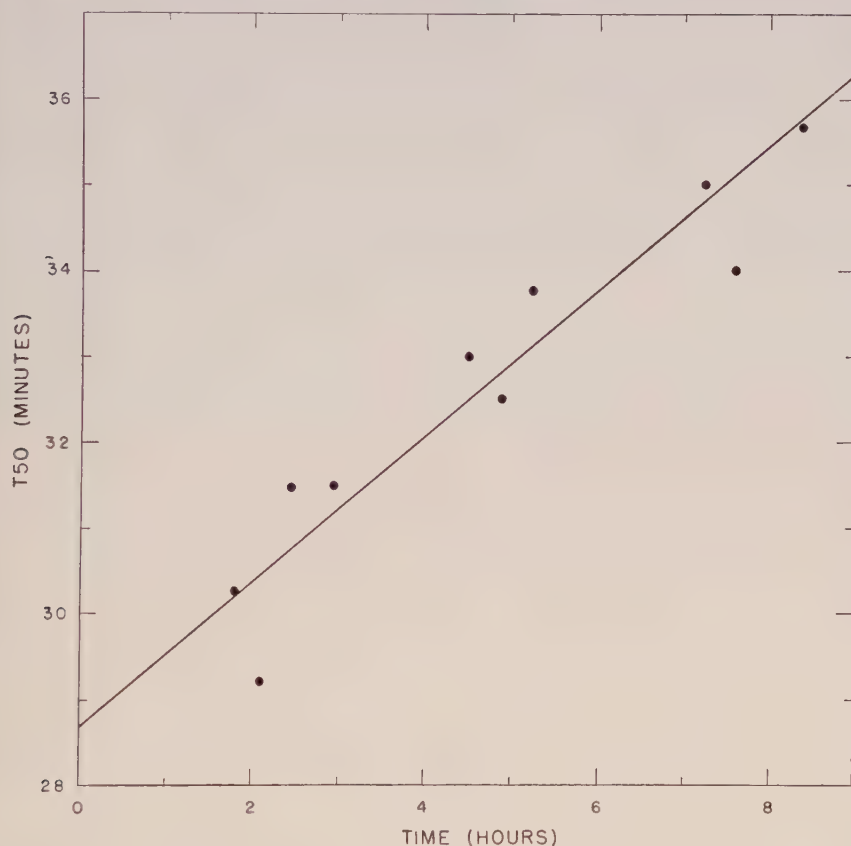


FIGURE 3. Increase in resistance of mosquito larvae to 0.1 p.p.m. heptachlor with time after removal from the culture medium. The slope of the line in this case was 0.84.

extend the total rearing time to 65 hours. For most work the use of a standard correction factor is more convenient.

The original observations on the day-to-day reproducibility of 44-hour larvae when tested against DDT at 0.1 p.p.m. gave an average value for T_{50} of 27.00 minutes with a standard deviation of 4.21 minutes (2). Midway during this work a second series of tests was run against 0.1 p.p.m. heptachlor on 22-hour larvae keeping the rearing conditions constant. The average value obtained on 21 batches tested over a period of 52 days

was 31.32 ± 3.19 minutes. Thus, the over-all batch-to-batch reproducibility had been improved by about 25 per cent in an equivalent T_{50} range during the intervening period. At the close of the program a third series of tests was run using the rearing conditions described in the Materials and Methods section (Table II). This time the average value for T_{50} was 29.14 ± 1.75 minutes at 0.1 p.p.m. heptachlor. When these data were corrected for time after removal from the nutrient, the standard deviation

TABLE II

REPRODUCIBILITY OF T_{50} TESTS AT 0.1 P.P.M. HEPTACHLOR OBTAINED ON DIFFERENT BATCHES OF LARVAE AND CORRECTED FOR TIME AFTER REMOVAL FROM NUTRIENT

Date obtained	T_{50} (minutes)	Time after removal (hours)	T_{60}^0 (minutes)
July 16	28.5	4.7	25.1
17	30.5	4.9	27.9
20	28.0	0.9	27.4
20	30.0	2.6	28.9
21	26.0	1.3	25.1
22	29.5	1.0	28.8
23	30.5	1.4	29.5
24	28.5	1.4	27.5
27	26.0	1.5	25.4
28	30.5	1.5	29.4
29	26.5	1.5	25.4
30	28.0	1.5	26.9
31	28.0	1.9	26.6
August 3	30.5	1.9	29.1
4	31.0	2.0	29.6
5	30.5	2.3	28.8
6	31.0	3.4	28.5
9	31.0	4.8	27.5
Average	29.14	—	27.63
s	1.75	—	1.58

was reduced to 1.58 minutes. This is in the same range of reproducibility obtainable on replicate tests made on the same batch (Table I), so no advantages can be expected on further improvements in larval uniformity unless these are accompanied by corresponding improvements in the test method itself. During the period covered by this investigation the standard deviation between batches was reduced from 4.21 to 1.58 minutes or about 62.5 per cent. This is a substantial improvement and should be helpful in correlating inhibition data obtained on larvae from different batches.

The hyperbolic concentration-inhibition relationship proposed for DDT, methoxychlor, and lindane on 44-hour larvae (2) also holds for heptachlor on 22-hour larvae. This, together with the regression characteristics of a number of other insecticides, will be discussed in a later publication.

FURTHER CAUSES OF VARIABILITY

The changes which have been made in rearing procedures have improved larval uniformity to the point where batch-to-batch variations are no greater than those obtained on retesting the same batch. This does not imply, however, that larvae can be produced indefinitely without encountering periods when the time of molting and yield of second instars fluctuate. As rearing methods have been improved, these intervals have become less frequent, but they still occur from time to time.

An important source of variation may be the type and degree of microbiological contamination. This would tend to change the composition of the rearing medium through the accumulation of products of bacterial metabolism. Some of these could supply growth factors while others might be harmful. The 22-hour rearing schedule reduces the time in which such changes can occur. Nevertheless the build-up of bacterial flora must be considerable, and it probably differs widely with the type and extent of initial contamination. Attempts were made to reduce bacterial growth through the incorporation of antibiotics in the culture media. Streptomycin was the most effective material tested, but when used at a concentration of 5.0 p.p.m. it inhibited growth of the larvae. The first instars failed to cast at 22 hours, and though the larvae were small, their resistance as measured by T50 was abnormally high. Whether this was caused by a direct effect of the streptomycin or was due to suppression of the bacterial flora has not been demonstrated. Efforts to maintain sterile cultures through sterilizing the eggs and autoclaving the medium met with variable results. Control of contamination was erratic and the larvae obtained from such cultures were usually small and did not cast into second instars during the required interval. It is reasonable to suppose that better uniformity could be obtained from sterile cultures maintained on a complete medium, but with existing techniques this would be difficult to accomplish on a large scale.

Wigglesworth (5) has been able to maintain fourth instar larvae of *Aedes aegypti* in a practically bacteria-free medium by adjusting the pH to 4.0 with a 0.01 *M* potassium hydrogen phthalate-phosphoric acid buffer. However, Woodhill (6) observed that the rate of development of *Culex fatigans* was slower in very acid or very alkaline media in the pH range of 4.2 to 9.0. Whether bacterial growth can be reduced successfully by adjusting pH without altering growth and resistance to toxicants has yet to be demonstrated.

The stability of the adult cultures is another factor which requires further attention. Variations in the average age and viability of the adults from which eggs are obtained might well be reflected in the development of the test larvae. During this work the adults were maintained in a constant temperature room and fed on a fixed schedule. The colonies were

replenished daily with newly emerged imagos. However, the average age in newly established colonies was probably less than in the older ones. It is uncertain whether the population ever becomes static under the conditions employed.

The importance of a blood meal to egg deposition has been amply demonstrated (1), and it is also likely that changes in feeding will affect the growth and viability of the embryos. The guinea pigs which are used as a blood source vary in temperament, and a restive animal may discourage feeding and lead to a light deposition of eggs. In any case there is evidence that larval viability undergoes periodic changes and this might be related to conditions in the adult colonies.

SUMMARY

A number of improvements have been suggested for rearing larvae of *Aedes aegypti* (L.) for use in the evaluation of insecticides by the photomigration (T50) test. Resistance to toxicants increases with age and instar, and there is a sharp drop in resistance at each molt. The best time to use larvae for bioassay is directly after the first molt when the time required for 50 per cent inactivation at a standard concentration of toxicant is lowest. Procedures are described for rearing larvae that will result in second instar larvae in 22 to 24 hours after introduction of the food supply. Any first instars which remain should be rejected. T50 results increase slowly after removal of the larvae from the culture medium. They should be corrected by subtracting 0.7 minute for each hour after removal.

The most important changes in rearing conditions over existing methods include preconditioning of eggs and newly hatched larvae, better temperature control, and mass rather than batch rearing. The inclusion of a surface active agent in the rearing and testing media eliminates experimental difficulties caused by the tendency of some larvae to float on the surface of the solution when exposed to insecticides. When reared under the conditions described the average value for T50 on 21 batches tested over a period of 52 days was 27.63 ± 1.58 minutes with heptachlor at 0.1 p.p.m. as the reference insecticide. The standard deviation previously obtained with DDT was 4.21 minutes; hence the changes described have considerably improved the reproducibility of the larvae with respect to resistance.

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Notes

PARTITION OF INSECTICIDES BETWEEN N,N-DIMETHYLFORMAMIDE AND HEXANE

H. P. BURCHFIELD AND ELEANOR E. STORRS

The presence of lipids in plant extracts inhibits the action of insecticides in bioassay tests (1, 2, 3). Jones and Riddick (3) suggest this effect can be minimized by extracting the insecticides from hexane solution with acetonitrile. They present data which show that considerable amounts of some insecticides accumulate in the more polar (acetonitrile) phase. Presumably most of the lipids remain in the hexane.

In connection with earlier work on a bioassay method using mosquito larvae (1), experiments with a number of solvents were carried out in these laboratories, and a N,N-dimethylformamide-hexane mixture was found to give the most favorable partitioning.

Ten ml. of *n*-hexane (b.p. 62°-67° C.) containing 0.1 g. of insecticide or lipid were shaken with 10 ml. of dimethylformamide (b.p. 152°-154° C.) in a 60-ml. separatory funnel, and the polar phase drawn off and analyzed. The lipid (corn oil) was determined gravimetrically by diluting the extract with water and re-extracting with hexane. The hexane was then washed with water, evaporated, and the weight of the residue determined. The chlorinated hydrocarbon insecticides were determined by titration of the chloride ion liberated on refluxing with sodium in isopropyl alcohol (4).

The results are compared in Table I to those obtained by Jones and Riddick on lindane and DDT. Since both sets of results were obtained using a 1:1 solvent ratio, it is evident that the dimethylformamide system results in a more efficient extraction of insecticide. This is particularly true in the case of DDT where only 57 per cent of the insecticide was recovered on a single extraction with acetonitrile compared to 91 per cent for dimethylformamide. Jones and Riddick did not present data on the partition

TABLE I
COMPARISON OF ACETONITRILE AND DIMETHYLFORMAMIDE
EXTRACTION METHODS

Material	Per cent in polar phase	
	Acetonitrile	Dimethylformamide
Lindane	86	99.7
DDT	57	91.3
Aldrin	—	79.7
Corn oil	—	7.3

of lipids, but it can be assumed that fats and waxes would tend to remain in the hydrocarbon phase.

When corn oil was used as a typical glyceride only about 7 per cent of it was extracted by dimethylformamide. Bioassay methods are so sensitive to traces of lipids that a more complete separation would be desirable. However, the partition coefficients are favorable in both directions, and it should be possible to set up a countercurrent distribution method that would provide a highly purified sample in comparatively few steps.

Materials such as chlorophyll, carotene, and sterols may also inhibit insecticidal action. Some of these could be removed by a preliminary chromatographic adsorption step, while additional clean-up procedures might have to be developed for others.

The high boiling point (152° – 154° C.) of dimethylformamide does not prevent its use since the insecticide can be recovered by diluting the polar phase with a large amount of water and re-extracting with hexane.

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THE IMPORTED LONG-HORNED WEEVIL

ALBERT HARTZELL

This wingless beetle was first reported from Yonkers, New York, in 1929, by Andrew J. Mutchler (3) of the American Museum of Natural History. The beetles at that time appeared in great numbers in the garden of W. M. Faunce in Colonial Heights, Yonkers. They were identified by L. L. Buchanan of the United States National Museum as *Calomycterus setarius* Roelofs. The species is indigenous to Japan and was first described by Roelofs (4) in 1873. In spite of the fact that the insect was first reported in the United States from Yonkers, infestations have been localized and there has been no widespread dispersal of the beetles due, no

doubt, to their wingless condition. Beetles and plants showing characteristic feeding injury have been observed by the writer every summer since August, 1946. The insect has been reported also from other localities in New York State. Destructive outbreaks have occurred in limited areas in Connecticut, New Jersey, and Maryland, with scattered records as far west as Iowa (1, 2).

The adult is a grey beetle (Fig. 1 B) about one-eighth inch in length. It eats out round notches in the margin of the leaf blades so that the leaves have a scalloped appearance (Fig. 1 A). Among the favored host plants are the following: Boston ivy (*Parthenocissus tricuspidata* Planch.), English ivy (*Hedera helix* L.), lespedeza (*Lespedeza* sp.), African marigold (*Tagetes erecta* L.), rose (*Rosa* sp.), and Virginia creeper (*Parthenocissus*



FIGURE 1. Imported long-horned weevil and characteristic feeding injury on viburnum.

A. (Left) Normal leaf. (Right) Feeding injury by adult beetle. B. Adult beetle.

quinquefolia Planch.). Other plants attacked are: chrysanthemum (*Chrysanthemum* sp.), clover (*Trifolium* sp.), day lily (*Hemerocallis flava* L.), deutzia (*Deutzia* sp.), firethorn (*Pyracantha* sp.), forsythia (*Forsythia* sp.), hollyhock (*Althea rosea* Cav.), iris (*Iris* sp.), maple (*Acer* sp.), milkweed (*Asclepias syriaca* L.), mock-orange (*Philadelphus* sp.), phlox (*Phlox paniculata* L.), privet (*Ligustrum ovalifolium* Hassk.), redtop (*Agrostis alba* L.), snowberry (*Symphoricarpos racemosus* Michx.), storksbill

(*Pelargonium* sp.), sweet potato (*Ipomoea batatas* Lam.), Swiss chard (*Beta vulgaris* L. var. *cicla* L.), and viburnum (*Viburnum* sp.).

The insect probably passes the winter as a partly grown larva, similar in habit in this respect to the Japanese beetle (*Popillia japonica* Newm.). Pupation occurs before the middle of June. The adult beetles appear from the latter part of June until October, but are not abundant until the last half of July. The beetles are attracted to light and colored surfaces and sometimes become a nuisance in dwellings.

The imported long-horned weevil can be killed by spraying with rotenone when the beetles appear on the foliage, although effective control measures have not as yet been fully perfected.

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A SIMPLIFIED PROCEDURE FOR MEASURING CELLULOSE DIGESTION BY RUMEN MICROORGANISMS¹

RICHARD HENDERSON, FLORENCE E. HORVAT, AND RICHARD J. BLOCK

One of the important functions of rumen microorganisms is the metabolism of cellulose to soluble carbon compounds which can be utilized by the ruminant animal. Several methods have been reported for determining cellulolytic activity of rumen flora *in vitro*. These include chemical determinations of cellulose when alfalfa leaf meal (6) or finely ground cellulose from wood (1, 7) is used as the cellulose source, visual observation of disintegration of cotton thread made by Hoflund, Quin, and Clark (cited by Doetsch and Robinson, 3), and the measurement of metabolic end-products when carboxymethyl cellulose is used (11). The chemical methods of determining cellulose in rumen contents are long and laborious. The other methods mentioned do not yield a direct, quantitative estimate of cellulose digestion.

A variety of methods has been devised for measuring cellulolytic activity of organisms from sources other than the rumen. O'Neill *et al.* (9) and Newcomb (8) evaluated cellulolytic activity of some basidiomycetes by measuring the time required for Cellophane strips to be digested to the point where a uniform load broke the strips. Tensile strength of cotton cloth treated with preservatives and exposed to fungus attack, either by soil burial or in pure culture, is used for evaluation of textile preservatives. Loss of weight of wood blocks exposed to fungus attack is used for evaluation of wood preservatives.

It was thought that an easy, direct method of evaluating cellulolytic activity of rumen microorganisms *in vitro* could be developed if a suitable cellulose source could be found. Such a cellulose source should have the following properties: (a) a form which can be utilized by rumen microorganisms but which is not too highly modified; (b) high wet-strength so that it does not disintegrate in liquid culture; (c) a form having a reasonable weight per unit area so that measurable weight loss can be obtained before disintegration occurs; (d) a purified form such that the cellulosic material does not introduce other materials into the culture.

Wood blocks cannot be used because rumen microorganisms do not utilize cellulose in the cellulose-lignin complex and because wood contains water soluble substances. Cellophane was tried and is easily digested because its cellulose is modified to a considerable extent. Cotton thread breaks

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into smaller pieces when only a small amount of the total weight has been digested. Some form of paper appeared to be the most likely choice. Several types of filter paper were tried but none was found which did not disintegrate on incubation in the liquid medium without the presence of cellulytic microorganisms. However, there are a number of high wet-strength parchment papers available, one of which was tried and found to be satisfactory.

MATERIALS AND METHODS

Fresh rumen contents obtained from slaughtered animals were packed in suitable containers and immediately frozen by mixing with dry ice. Dry ice was preferred for freezing since it not only accomplished the freezing very quickly but also maintained the material under anaerobic conditions while it was being frozen.

A basal medium consisting of urea, glucose, and a modification of the inorganic salts used by Burroughs *et al.* (2) was used. The composition of the medium was as follows: NaH_2PO_4 , 2041.6 mg.; NaHCO_3 , 2041.6 mg.; KCl , 262.4 mg.; KI , 28.03 mg.; NaCl , 262.4 mg.; MgSO_4 , 87.5 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 31.2 mg.; MnSO_4 , 15.6 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.6 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.47 mg.; CoSO_4 , 0.70 mg.; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.07 mg.; $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 0.16 mg.; K_2CrO_4 , 0.08 mg.; NaAsO_3 , 0.13 mg.; glucose, 500 mg.; urea, 840 mg.; water to make 1 liter. Stock solutions of the individual salts were used to make up the basal medium as required. It was not sterilized prior to use as a massive inoculum was used. Parchment paper strips (Patapar 27-47, Paterson Parchment Paper Co., Bristol, Pa.) were used as the added cellulose source.

Four hundred and fifty milliliters of basal medium were heated to 55° to 60° C. and placed in a Waring Blendor. Carbon dioxide was bubbled through the liquid while 200 g. of frozen rumen contents were added and thoroughly blended. The rumen contents should not be allowed to thaw before being blended and should be warmed to 39° C. immediately after blending. The blended material was filtered through cheesecloth. The filtrate was divided into aliquots, one being used for control cultures and test substances added to the others.

Strips of parchment paper were folded into one-quarter inch pleats, weighed, and placed in 19×150 mm. bacteriological test tubes. In some experiments the strips were 57 mm. \times 105 mm. and weighed approximately 270 mg. but strips 57 mm. \times 210 mm. weighing approximately 540 mg. were used for most of the experiments. Fifteen ml. of culture were added to each tube, the tubes flushed with carbon dioxide and closed with stoppers fitted with Bunsen valves. The Bunsen valve allowed the escape of gases formed during incubation but prevented entrance of air. The tubes were incubated at 39° C. in an air incubator. At the end of the incubation

the paper strips were removed, washed in running water, air dried, and weighed. The liquid was used for analyses for metabolic end-products.

RESULTS AND DISCUSSION

As much as 40 per cent of the weight of a strip was lost by digestion and the strip had retained sufficient strength to allow washing, drying, and weighing. In general 10 to 20 per cent of the initial weight was digested in 18 to 36 hours depending on the rumen sample. It was found desirable to run 10 to 15 tubes in each group in order that differences of a few per cent be statistically significant.

A large number of materials have been tested for stimulation of cellulose digestion by rumen cultures. The results of two experiments are presented as examples of the method.

In one experiment $MgSO_4$ was omitted from the basal medium and added at the usual level and at four times the usual level to aliquots of the filtrate. The paper strips used in this experiment were 57 mm. \times 105 mm. and had an initial weight of approximately 270 mg. each. The cultures were incubated for 24 hours. The loss in weight of the paper strips is given in Table I. The averages for each group and the limits for 95 per cent confidence interval are listed. The initial pH of each aliquot and the final pH of the pooled cultures of each group are also tabulated.

The above results were obtained while the method was being developed.

TABLE I

LOSS IN WEIGHT OF PAPER STRIPS (MG.) RESULTING FROM CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO AS INFLUENCED BY CONCENTRATION OF $MgSO_4$ OR PERIOD OF INCUBATION

Concentration of MgSO ₄ , mg./l. (24 hours' incubation)		Period of incubation, hours (87.5 mg. MgSO ₄ /l.)			
87.5	340	17	21	24	
39.6	6.3	34.3	58.5	68.0	
34.8	7.9	34.0	61.4	65.6	
28.4	8.4	35.0	61.9	66.3	
24.7	9.3	32.3	60.5	67.3	
24.6	16.5	39.3	53.5	64.7	
30.7	11.2			68.8	
24.5	13.7			69.4	
33.9	11.7			65.9	
29.9	22.8			65.4	
18.4	21.3			61.2	
14.1	5.5				
21.7	6.4				
18.6	4.8				
25.6	4.9				
	5.3				
Averages	26.4 ± 3.9	10.4 ± 3.7	35.0 ± 3.8	59.2 ± 3.8	66.3 ± 2.0
Initial pH	7.1	7.0	6.7	6.7	6.7
Final pH	6.6	6.9	6.5	5.8	5.5

There is a wide variation in the weight loss of strips within a group. Such variation was reduced with improvement in technique and experience in the method.

The second experiment was carried out to determine the effect of time of incubation on cellulose digestion. Groups of cultures were incubated for 17, 21, and 24 hours. The paper strips used were 57 mm. \times 210 mm. and weighed approximately 540 mg. each. The MgSO_4 level was that listed for the basal medium. The loss in weight of paper strips, the averages, and the limits for 95 per cent confidence interval are given in Table I. The cultures from the tubes in each group were pooled at the end of incubation and the pH of each pooled sample is listed. The variation of weight loss within each group is sufficiently small so that differences of a few per cent between groups are significant with the number of tubes used.

From the above data it was calculated that the average rate of loss of weight was 2.06 mg. per hour for the first 17 hours, 3.50 mg. per hour for the four hours from 17 to 21 hours, and 2.33 mg. per hour from 21 to 24 hours. The variation in the rates of digestion may be due to changes in the pH of the medium caused by the formation of organic acids. It is known that acids and other metabolic end-products may inhibit cellulose digestion. Gall and Glaws (5) used a permeable type of artificial rumen through which metabolic products diffused out and new nutrients diffused in from a large volume of medium. Such a procedure more nearly duplicates *in vivo* conditions for rumen microorganisms. Since comparative data are obtained by running controls along with test material, no attempt was made to adjust pH during incubation in the method described here. However, in many types of experiments it is desirable to obtain values for several incubation times.

It is recognized that there may be changes in the predominant species from the start of incubation to the end. Pearson and Smith (10) have shown that rumen cultures change in character of predominant types after 6 to 8 hours' incubation *in vitro*. Burroughs *et al.* (2) point out that end-products are not controlled in the same manner and that different types of organisms may develop in the *in vitro* culture as compared to *in vivo*. Other disadvantages of *in vitro* techniques stated by Elsden (4) are that end-products are already present in the inoculum and so are nutrients which allow metabolism in the absence of added substrate. Recognizing the fact that the method presented does not duplicate *in vivo* conditions of rumen microorganisms, it does furnish a relatively simple, rapid technique for screening a large number of materials for stimulation of cellulose digestion by rumen microorganisms. Data obtained from any *in vitro* method must be checked by field trials before their value in practical ruminant feeding can be determined.

In addition to use in studies of rumen microbiology, parchment paper

may prove a good cellulose source in studying cellulolytic activity of other organisms.

SUMMARY

A method is presented for the study of cellulose digesting activity of rumen microorganisms *in vitro*. The cellulolytic activity is measured by the loss of weight of parchment paper strips incubated in cultures of rumen microorganisms in standard bacteriological test tubes. Materials can be evaluated for stimulatory or inhibitory action on cellulose digestion by addition of such materials to aliquots of the culture rumen contents. The parchment paper strips used in this method may be a useful source of cellulose in studying cellulolytic activity of other microorganisms.

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A METHOD FOR OBTAINING PIERCING-SUCKING MOUTH PARTS IN HOST TISSUE FROM THE TARNISHED PLANT BUG BY HIGH VOLTAGE SHOCK

MYRON C. LEDBETTER AND FLORENCE FLEMION

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), (6) severely damages many different species of economic plants. To study the manner of penetration and mode of travel of the piercing-sucking mouth parts (stylets) in host tissue, it was essential that stylets *in situ* be obtained. Various attempts at killing *Lygus* with stylets inserted into the tissue by the methods used for aphids, leafhoppers, and other insects, such as molten wax (7), anesthetic (1, 4) and killing solution (3, 8), were unsuccessful since *Lygus* bugs quickly withdraw their stylets at the slightest provocation. Several stylets *in situ* were obtained, however, by gradually lowering the temperature until the insects were so inactive that ether could be used without causing the insects to withdraw the stylets from the tissue, but this was a very painstaking procedure which resulted in many failures. It was not until the electrical apparatus¹ described below was assembled that adequate material was obtained for histological study (5).

The equipment consisted essentially of an automobile ignition system in which the distributor and spark plugs were replaced by a knife switch and wire electrodes. A 6-volt direct current supply was used with a spark coil, knife switch, and a capacitor connected as shown in Figure 1. The spark was initiated by opening the knife switch. It was necessary to close the switch before attaching the electrodes to the terminals, otherwise the insect (Fig. 2 G) was disturbed by the induction current produced as the switch was closed. On opening the switch the current was sufficient to stun or kill the insect. A detailed explanation of this type of circuit is given by Crouse (2, pp. 250-259).

To obtain stylets *in situ* in green beans (cultivated varieties of *Phaseolus vulgaris* L., obtained in local vegetable markets) pieces of pod about 30 to 40 mm. long were covered with masking tape except for a small exposed feeding area (Fig. 2 B). The electrodes, two pieces of thin copper wire (B & S gauge 26) each about 12 cm. long, were taped to this bean pod with the wires on opposite sides of the feeding area exposed and at their maximum proximity, about 2 mm. (Fig 2 A, C), so that the insect had to stand on or near these electrodes to feed. The taped bean pod and the insect

¹ The authors are indebted to Dr. Richard C. Back of Ethyl Corporation, New York, N. Y. for suggesting electrical means for obtaining stylets *in situ* and to Mr. Robert P. Ledbetter of Workshop for Electronics, Oklahoma City, Okla. for suggesting the circuit used in the apparatus.

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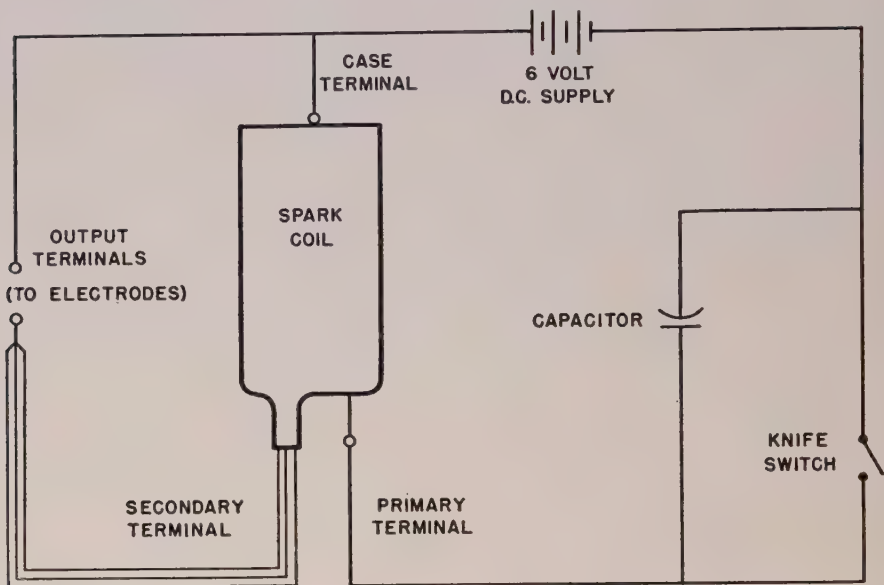


FIGURE 1. Circuit diagram of apparatus used to produce high voltage shock.

were placed in a glass vial with the two electrodes extending beyond the cork stopper (Fig. 2 C). The insects were conditioned to locate the feeding area by providing them with similar tissue taped in this manner for about ten days prior to the high voltage shock.

The electric shock was found to be most effective when legs on opposite sides of the body were in contact with the two electrodes. With the insect in this position the electrodes were attached to the output terminals (Fig. 1) after closing the knife switch. The insect was then observed with a low power (10 X) stereoscopic microscope. At the moment of deepest penetration into the tissue, as indicated by the downward stroke of the head, the knife switch was opened to produce the high voltage shock. Since the electric shock usually stunned rather than killed the insect, ether on cotton was introduced immediately into the glass vial containing the insect and tissue. Afterwards the stylets were severed with a small sharp pointed pair of dissecting scissors or seared off with an electrically heated nichrome wire. The portion of the bean pod tissue with stylets *in situ* was then placed in fixing solution for subsequent histological study.

The technique for obtaining stylets in umbelliferous fruits was similar except that the peduncle of fennel (*Foeniculum dulce* Mill.) fruit was taped to the tip of a glass rod as shown in Figure 2 D, E, F, and the electrodes were placed over the fruit so that their greatest proximity was in the region of the insect's insertion of stylets into the fruit.

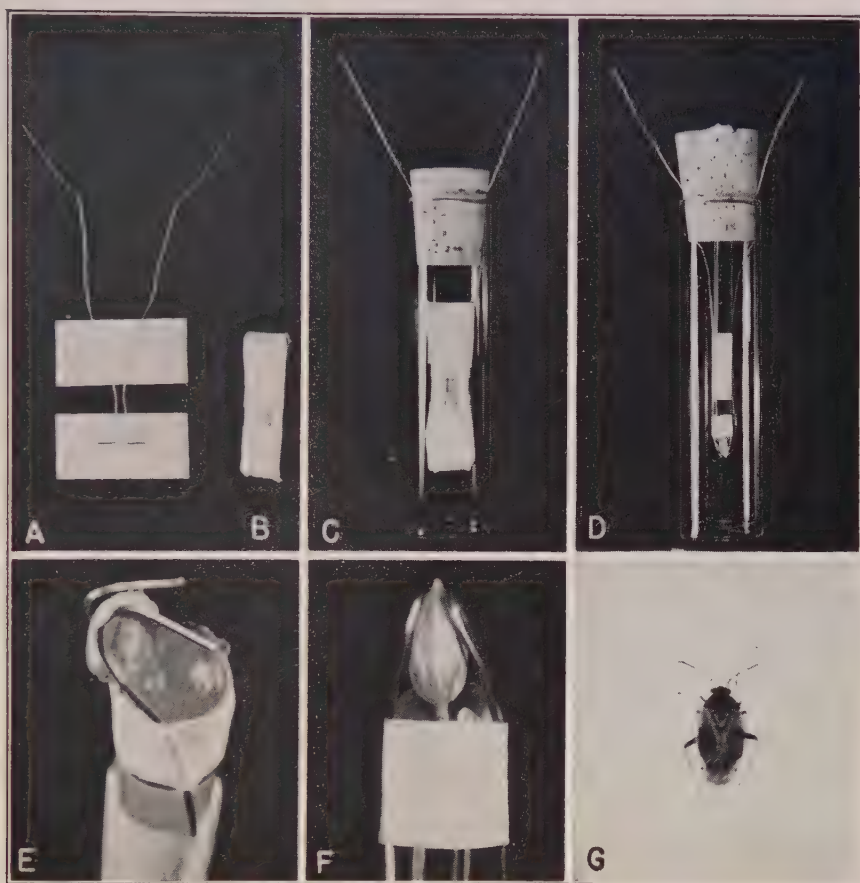


FIGURE 2. Position of electrodes (A) on tape prior to being placed on taped bean pod (B). (C) Bean pod in stoppered vial with electrodes exposed over feeding area. (D) Fennel fruit with electrodes in position. (E, F) Closeup views of (D). (G) Adult *Lygus*. A, B, C, D ($\times 0.5$). E, F, G ($\times 3.5$).

With some modifications this technique may find wider application in obtaining stylets from many other kinds of insects *in situ* in host plants or animals (if anesthetics are used) since simpler techniques do not act as quickly as electrical shock.

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PENETRATION AND DAMAGE OF PLANT TISSUES DURING FEEDING BY THE TARNISHED PLANT BUG
(*LYGUS LINEOLARIS*)

FLORENCE FLEMION, MYRON C. LEDBETTER, AND ELIZABETH
SCHAEFER KELLEY

Various species of *Lygus* are distributed throughout the world and severely damage a wide range of economic plants as well as many weeds and grasses (13). Their feeding behavior of piercing and sucking plant tissues produces adverse effects on growth (1, 5, 18), destruction of flowers (3, 11, 12, 16, 20), reduction of seed yield (4, 7, 10), and embryoless seeds (8). Since injury following feeding is so extensive, various investigators have postulated that some toxic material may be involved (3, 18). Studies with the use of radioactive phosphorus (P^{32}) have shown that there is a transfer of oral material from insect to host during feeding by *L. lineolaris* and the quantities deposited have been estimated (6). However, it has not yet been shown that such secretion is phytotoxic. In regard to the withdrawal of cell fluids from plant tissues Awati and Wolfe-Barry (2) studied the structure of the head and stylets (piercing-sucking mouth parts) of *L. pabulinus in situ* and visualized the coordination of the various muscles and structures, from the piercing of the tissue to the digestion of fluids in the stomach (mid-gut) as the mechanics of suction.

The purpose of the present investigation was to determine the manner of penetration and the subsequent damage of host tissue by the stylets of the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) [*L. oblineatus* (Say)] (17). The movements of the stylets when feeding on bean pod, carrot, and beet root tissue were observed directly with the aid of the microscope and were also recorded on motion picture film.¹ *Lygus* bugs were killed while feeding on bean pod and parsnip seed and the tissues with stylets *in situ* were subsequently studied. When observed visually the stylets moved rapidly in all directions and when seen in histological material they traveled intra- as well as intercellularly. Cavities, collapsed plasmolyzed cells, and light-staining cell walls were observed in the necrotic lesions which developed after feeding.

MATERIALS AND METHODS

The studies were conducted with adult *Lygus* which had been collected locally or which had hatched from eggs laid by collected insects. These insects were held at either room temperature or 15° C. (59° F.) and maintained until used on green bean (*Phaseolus vulgaris* L.) pods. *Lygus* also

¹ This film was shown at the Eastern Branch Meeting of the Entomological Society of America, Philadelphia, Pa., Nov. 16, 1953.

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thrived when maintained on root tissue of either beet (*Beta vulgaris* L.) or carrot (*Daucus carota* L. var. *sativa*).

Feeding observations. When bean pod was used, the insect with a thin section of mature pod was placed in a glass microcell which consisted of a slide (25×75 mm.), a microslide ring (18×10 mm.) taped to the slide, and a cover glass (25×25×1 mm.) cut from a microscope slide. With either beet or carrot, a free-hand section about 5×5 mm. and approximately 80 microns (a few cells) in thickness was placed in water immediately after sectioning and then attached with masking tape to the underside of the cover glass of the microcell containing the insect. The tape not only held the material in position but prevented the rapid dehydration of the tissue which was entirely covered with tape except for a narrow strip at one end through which the stylets could enter. When in the tissue (between glass and tape), the brown stylets could be readily seen with the stereoscopic microscope (10 to 30×).

Stylets in situ. By using an electrical apparatus described elsewhere (14) 34 stylets *in situ* in bean pod were obtained within a few weeks. Immediately following the electric shock and subsequent killing of the insect by ether, the stylets *in situ* were severed from the body of the insect. The plant tissue was then trimmed and placed in either formalin-propionol-alcohol (FPA) or Randolph's modified Navashin fluid (Craf). Subsequently 11 were cleared with sodium hydroxide (9, p. 216) while 23 were sectioned and stained.

The electric shock technique was also used to obtain 14 stylets *in situ* in immature seeds of parsnip (*Pastinaca sativa* L.). This material as well as bean pod and fennel leaf, upon which *Lygus* had fed, was processed for histological study. In the case of tissue damaged from feeding, the material was held for 2.5, 6, 24, and 48 hours in a moist condition at room temperature prior to being trimmed and placed in the fixing solution.

Histological technique. Serial sections were cut at 10, 15, or 20 microns from the material embedded in rubberized paraffin.

Most of the staining was done in Harris' hematoxylin (15, p. 58) and safranin. A 1 to 2 per cent ferric chloride or ferric ammonium sulfate solution in 50 per cent alcohol was used as a mordant prior to the hematoxylin, since it was found that these ferric solutions allow heavy staining of the cell walls, and produce good material for photomicrographs. After 10 minutes in the mordanting solution the slides were washed well in 50 per cent alcohol and stained for two to five minutes in Harris' hematoxylin. Iron hematoxylin and safranin were used for some of the material.

Motion pictures. A film illustrating the feeding mechanics of the tarnished plant bug has been produced at this Institute.² When photographing

² The cinephotomicrography was supervised by Mr. William G. Smith, Jr. of the Illustration Division.

the movements of the stylets in host tissue, the technique used was that described above for observing stylets when feeding in thin sections of carrot root tissue taped to glass. Also shown in the film are photomicrographs of histological material (described below) such as stylets *in situ*, tissue breakdown following feeding, etc.

RESULTS

FEEDING MECHANICS

The mouth parts. The piercing-sucking organ (Fig. 1 B) consists of a pair of interlocked inner (maxillary) stylets encased in a pair of mandibles (outer stylets) and as shown in Figure 1 G the tip of the inner stylets is smooth and lancet-like while the mandibles are barbed. For more details see Awati and Wolfe-Barry (2) who studied the arrangement of the different structures of the head of *Lygus pabulinus*.

Action of stylets. The movements of the fine, needle-like, chitinous stylets of the tarnished plant bug were observed in thin sections of green bean pod tissue through the microscope. The extremely flexible stylets moved rapidly in various directions with frequent plunging and withdrawal and appeared to be following no particular path. This irregular route could be followed more closely when sections of either carrot or beet root tissue only a few cells in thickness were taped to glass (Fig. 1 A). With each mandible (Fig. 1 B) capable of independent protraction and retraction, the stylets travel in the tissue by short rapid thrusts and bend in any direction such as turning back or making a right angle. These various movements have been recorded on motion picture film and an enlarged photograph of one frame of the 16 mm. film appears in Figure 1 A.

In addition to following the various movements of the stylets it was also possible to observe with the microscope the removal of the contents of individual cells of beet root. In their search for nutrients the insects sucked up the red pigments which were later excreted. The emptying of a given cell was so extremely rapid that it was difficult to follow the actual process but the cell before and after removal of the pigments was visible evidence that feeding had occurred.

HISTOLOGICAL STUDIES OF STYLETS IN SITU IN BEAN POD

When bean pod tissue which had been cleared with sodium hydroxide was examined in polarized light, the stylets *in situ* could be readily followed from the surface down into the deeper tissues. Usually, as noted above, the route taken was irregular. With paraffined material, cross sections of the stylets in the various tissues were readily obtained and occasionally a considerable portion of the stylets was procured in one section as shown in Figure 2 A.

Entry of stylets. When at rest, the stylets lie in a groove in the labium.

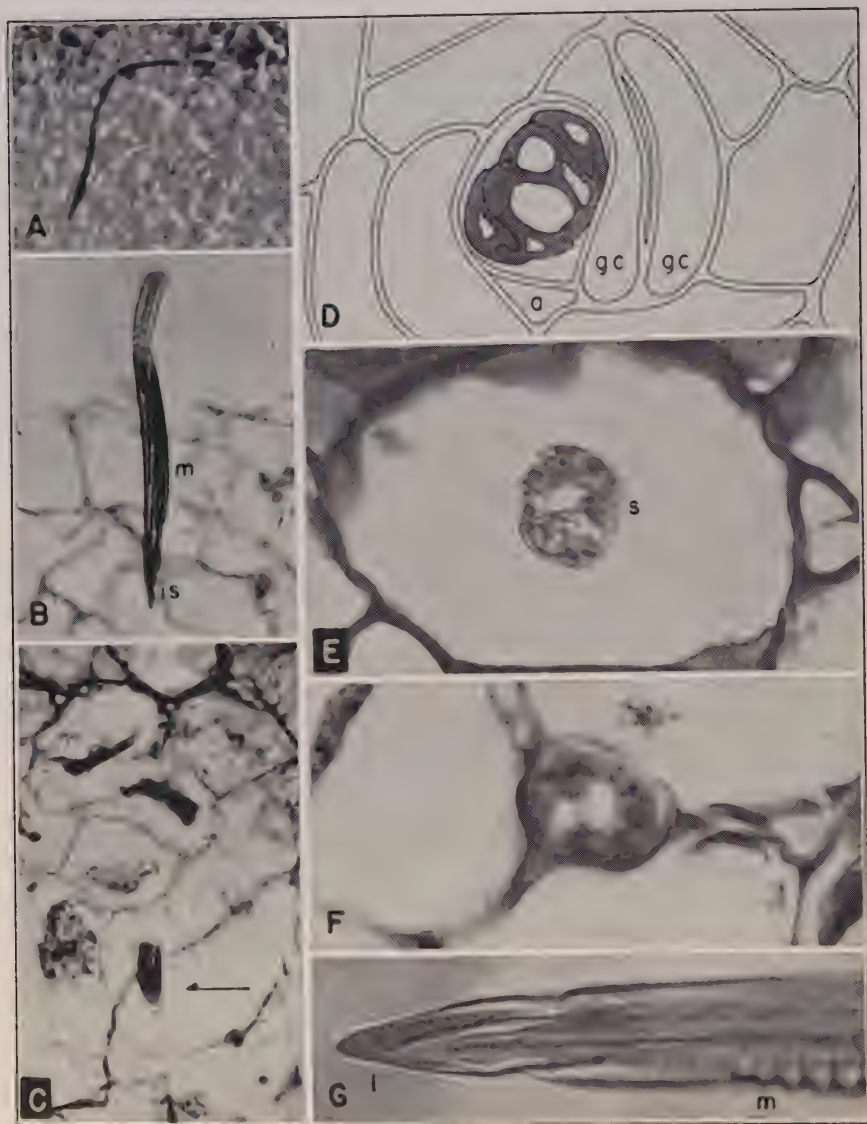


FIGURE 1. Photomicrographs and camera lucida drawing (D) of *Lygus* stylets in tissue. A (fresh carrot); B to G (paraffin-sectioned bean pod); A ($\times 56$); B and C ($\times 200$); D ($\times 1000$); E ($\times 795$); F ($\times 900$); G ($\times 890$). A—stylets in position for feeding. B—tip of stylets in inner mesocarp (m=mandibles; is=inner stylets). C—tip of stylets in outer mesocarp near region of diagonal fibers. D—stylets (shaded) in epidermis between guard cell (gc) of stoma and adjacent cell (a). E and F—stylets in (s) and between cells of outer mesocarp. G—tip of stylets (l=lancet-like; m=mandible).

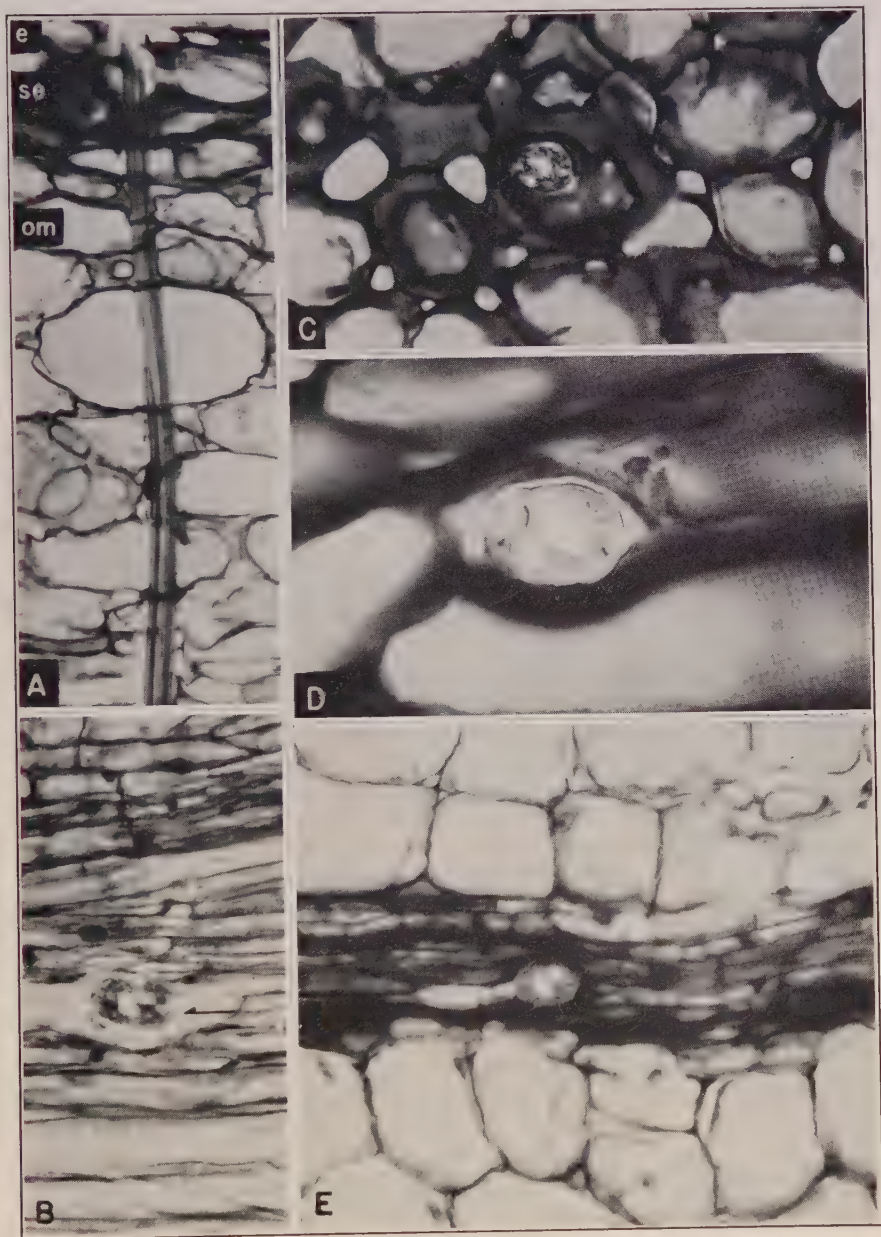


FIGURE 2. Photomicrographs of *Lygus* stylets in paraffin-sectioned bean pod. A ($\times 200$); B, C, and E ($\times 420$); D ($\times 900$). A—stylets in epidermis (e), sub-epidermal layer (se), and outer mesocarp (om). B, C, D, and E—stylets in diagonal fibers, outer mesocarp, sub-epidermal layer and vascular strand, respectively.

In selecting the point of entry, the sensory hairs at the tip of the labium probed the surface carefully before the stylets were forced into the bean pod tissue. In several instances the stylets entered the epidermis either by passing through the cells adjacent to the guard cells of a stoma or by going between these cells as illustrated in the camera lucida drawing (Fig. 1 D).

Stylets travel intra- and intercellularly. The stylets do not elongate; however, the labium can be bent and thus the deeper the penetration in the tissue the greater the bend in the labium. When penetrating the various tissues of the bean pod, the stylets traveled between (Fig. 1 F) as well as through the cells (Fig. 1 E-s) by puncturing the cell walls (Fig. 2 C and D). Sometimes the stylets took a somewhat straight path (Fig. 2 A); however, the route usually followed no particular pattern. In the photomicrograph of both the subepidermal layer (Fig. 2 A and D) and the outer mesocarp (Fig. 1 E and F and Fig. 2 A and C), it is seen that in a given type of tissue the stylets traveled inter- as well as intracellularly. The stylets in passing through the intercellular spaces force the cells apart as shown in the illustrations of the outer mesocarp (Fig. 1 F) and the vascular region (Fig. 2 E).

Although the tip of the stylets may reach the inner mesocarp (Fig. 1 C) this does not necessarily indicate a preferred feeding area for, as will be shown below, feeding damage may occur in any one of the various tissues of the bean pod. In all likelihood variety as well as texture and maturity of the pod (16) determine somewhat the extent of travel when locating a feeding area.

TISSUE BREAKDOWN FOLLOWING FEEDING

From the structure of the mandibles (Fig. 1 B and G) and the activity of the stylets in host tissue as described above it is not difficult to visualize that a great deal of mechanical injury can occur during the feeding process. In addition to laceration of the tissues and removal of cell fluids, other factors such as those arising from the deposition of oral secretions or introduction of microorganisms may also contribute to tissue breakdown.

Bean pod. Cavitation may occur such as that shown in the spongy parenchyma cells of the outer mesocarp (Fig. 3 B), in the region of the vascular bundles (Fig. 3 E), and in the parenchyma cells of the inner mesocarp (Fig. 3 A). Holes made by the stylets may occasionally be seen in the closely packed diagonal fibers which lie between the outer and inner mesocarp as shown in Figure 3 C. Cells adjacent to the collapsed cells in the damaged areas were found to be in various stages of disorganization; that is, plasmolyzed cells and walls that stained lighter and appeared at times to be thinner (Fig. 3 A, D, and E). This phenomenon was also observed in tissue with stylets *in situ* (Fig. 1 C and Fig. 2 B). Damage at the surface (Fig. 3 F) was usually slight when compared with internal break-

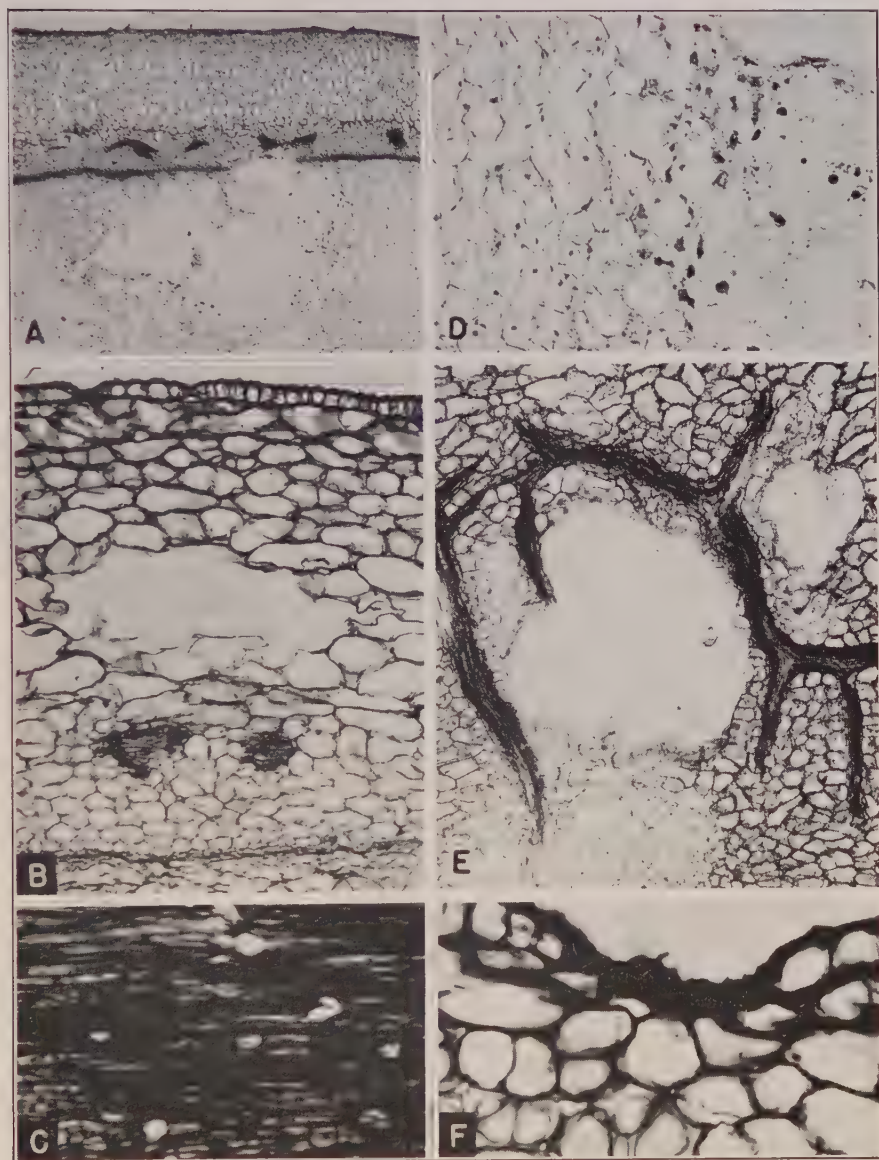


FIGURE 3. Photomicrographs of damaged bean pod tissue. Hours after *Lygus* feeding: 2.5 (A, D); 6 (C, E); and 24 (B, F). A ($\times 20$); E ($\times 50$); B and D ($\times 84$); C and F ($\times 200$). A, B—breakdown in inner and outer mesocarps, respectively. C—holes in diagonal fibers. D—closeup of plasmolyzed cells in A. E—destruction in vascular region. F—surface injury.

down. In these histological studies, neither feeding tracks nor any other indication except holes (Fig. 3 C) were obtained in the path taken by the stylets when traveling from the surface to the feeding area. However, the route taken in the tissue could be readily followed when stylets *in situ* were obtained.

Fennel leaf. When *Lygus* bugs feed on immature stem tissue near the growing tip, considerable injury occurs such as wilting, die-back and general debilitation of the plant (5). Forty-eight hours after feeding, immature petiolule tissue was processed and subsequently studied histologically. As seen from the collapsed cells in Figure 4 G, a great deal of destruction occurred in the chlorophyllous parenchyma cells immediately below the epidermis. The appearance of normal undamaged tissue is shown in Figure 4 E. An intermediate stage of destruction in this region is shown in Figure 4 F.

STYLETS IN PARSNIP FRUIT

By using the electric shock technique mentioned above, stylets *in situ* were readily obtained in parsnip fruits. The tissue was then processed for histological examination. A cross section of a typical green immature fruit as used in this study is shown in Figure 4 H. The stylets did not seem to select any specific area when entering the fruit coats since they entered at the tip (Fig. 4 A) in the region of the style (floral parts), along the side near the edge (Fig. 4 D), and in the general region of the ovule (Fig. 4 B). In this latter instance, the stylets passed alongside of the ovule and continued on (not shown) almost to the commissure. Since the stylets were found near only one ovule of the 11 fruits examined, this may indicate that *Lygus* bugs either feed on various tissues of these fruits and/or locate the ovule in a haphazard manner. The stylets appear to have no difficulty in traveling through the various tissues in the fruit coats and that they can penetrate deeply is shown in Figure 4 C where they have reached the central bundle of the commissure.

DISCUSSION

Various investigations concerned with the damage produced by *Lygus* in alfalfa (4, 10, 11), bean (3, 16), cotton (12), guayule (1), and potato (19) have shown that in each instance injury was restricted to the feeding area. Different stages of disintegration were described, that is, injury ranging from small lesions involving only a few cells to large necrotic areas in various stages of disorganization. It has been assumed by some of these investigators that the actual mechanical injury produced by *Lygus* during feeding was slight and that the severe damage which followed was due to the combined effect of withdrawal of cell fluids and deposition of a toxic substance. Although the actual deposition of saliva by *Lygus* during feed-

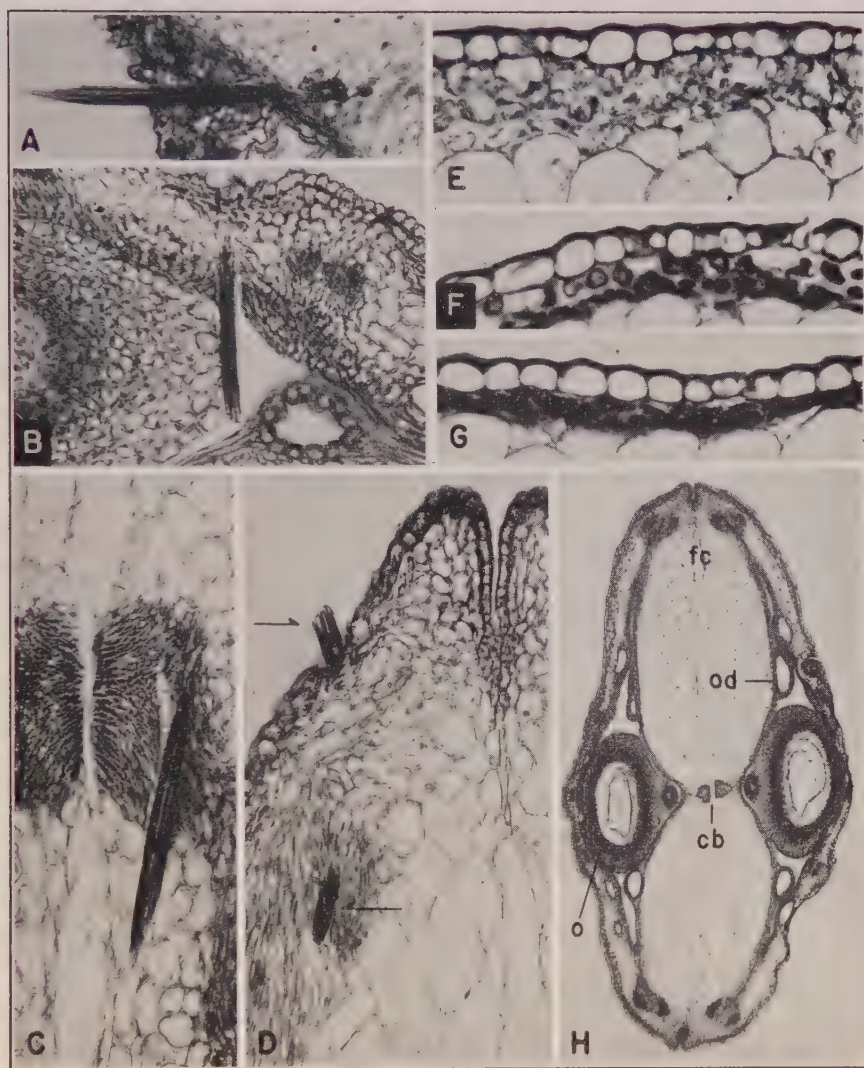


FIGURE 4. Photomicrographs of parsnip fruits with (A, B, C, D) and without (H) *Lygus* stylets *in situ* and of fennel petiolule tissue showing normal (E) and *Lygus* damage 48 hours after feeding (F, G). A, B, C, D ($\times 164$); E, F, G ($\times 200$); H ($\times 35$). A, D—stylets entering fruit coats through style and at outer edge of fruit wall, respectively. B—stylets bypassing ovule. C—stylets in central bundle of commissure. D—stylets in epiderm and vascular strand of fruit coat. E—normal tissue. F and G—intermediate destruction and collapse of chlorophyllous parenchyma cells, respectively. H—schizocarp (2 carpels): fruit coats (fc); oil duct (od); central bundles of commissure (cb); ovule (o).

ing has been observed (as shown in the motion picture film) and the amounts remaining in the damaged tissue have been estimated (6) it has not yet been determined whether such secretion produces secondary infections and/or is phytotoxic. By observing the movements of the stylets during feeding it was found that considerably more mechanical destruction takes place than had been postulated. A great deal of damage occurs as the needle-like, extremely flexible, and rapidly-moving stylets pass through and between the cells in the process of rupturing and emptying many cells.

SUMMARY

The mechanics of feeding by the tarnished plant bug, *Lygus lineolaris*, were observed directly through the microscope and were also recorded on motion picture film. The stylets (piercing-sucking mouth parts) not only moved rapidly when in plant tissues in search of food but frequently plunged into and withdrew from the tissue, traveled in various directions, and followed no particular path.

Photomicrographs of histological material illustrate that the stylets penetrated the various tissues of the bean pod and traveled intra- as well as intercellularly. In several instances the stylets entered the surface of the bean pod by piercing the epidermis adjacent to the guard cells of a stoma. Studies on mode of entry and travel in parsnip fruits indicate that the stylets readily pass through the various structures of these fruits.

Histological studies of the breakdown of bean pod and fennel leaf tissue following feeding revealed that many cells in the feeding area were affected—some had collapsed, while others were in various stages of disorganization.

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ROOT ROT OF LAWSON CYPRESS AND OTHER ORNAMENTALS CAUSED BY PHYTOPHTHORA CINNAMOMI^{1,2}

D. C. TORGESON³

Varieties of Lawson cypress or Port Orford cedar, *Chamaecyparis lawsoniana* Parl. are widely used in hedge, specimen and windbreak plantings in western Oregon. In the late 1930's a species of *Phytophthora* was found (9, 10) to be the cause of a serious root rot of Lawson cypress varieties in a number of plantings. The causal organism was subsequently described and named (20) *Phytophthora lateralis* Tucker & Milbrath. *P. lateralis* has become widely disseminated (18) and is one of the major causes of disease losses in Oregon nurseries. In 1951, the author reported (16, 17) that *P. cinnamomi* Rands (14) was also the cause of a root rot of Lawson cypress with symptoms identical to those caused by *P. lateralis*. *P. cinnamomi* had not previously been reported as occurring in Oregon. It is known to attack numerous ornamental and crop plants in a number of tropical, subtropical and temperate areas of the world (15). At present *P. cinnamomi* has a limited distribution in Oregon as compared to *P. lateralis* (18).

Shortly after *P. cinnamomi* was found in Oregon studies were initiated to obtain information on the epiphytology and etiology of the diseases caused by this fungus. Such information is necessary before effective control measures can be devised. *P. cinnamomi* was found attacking several plant species in a number of Oregon nurseries. Potentially it appears to be a very serious pathogen as it has a wide host range, is readily disseminated, and at present cannot be effectively controlled. Spread and development of *P. cinnamomi* root rot was found to be influenced by such factors as moisture, texture, and temperature of the soil.

MATERIALS AND METHODS

GENERAL METHODS

Isolation of the pathogen. Because of the difficulty with which *P. cinnamomi* was isolated directly from diseased tissues the method of Tucker

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(19), using apple fruits as a differential medium, was adapted for use. Sections of diseased stems or roots were surface sterilized for one to two minutes in 20 per cent commercial Clorox, rinsed in sterile distilled water and inserted into opposite sides of sound apples. The points of inoculation were then covered with Cellophane tape. The inoculated apples were incubated at room temperature. *P. cinnamomi*, if present, grew into the apple and within a week to 10 days had produced an area of firm dry rot around the point of inoculation. Aseptic transfers of decayed apple tissue were made from the margins of the brownish infected areas to plates of potato-dextrose-agar and incubated at room temperature for two to three days. The plates were then examined for the presence of the fungus.

The pathogen was isolated from soil using the method developed by Campbell (2) for isolating the fungus from under littleleaf-diseased and healthy shortleaf pine. Soil samples were collected and a portion of each sample packed into a one-half inch hole bored with a cork borer diagonally into but not quite through a sound apple. The hole was filled within about one-half inch of the top and distilled water added until the soil was well saturated. The opening was then sealed with Cellophane tape and the apples incubated at room temperature. Each soil sample was tested in duplicate. *P. cinnamomi*, if present, caused a firm dry rot of the apple tissue within 5 to 10 days and was readily isolated in pure culture from the infected areas.

Culture of the pathogen. All cultures of *P. cinnamomi* were maintained on potato-dextrose-agar slants. Pea broth was used as a medium when growing the fungus for inoculations and sporangial production studies. It was prepared by boiling one pound of dried peas for 10 minutes, straining off the liquid and adding sufficient water to the liquid to make a volume of 3 liters.

As the fungus does not produce sporangia on standard media, a method developed by Mehrlich (7) was used. The fungus was grown in pea broth for one week, the mycelia removed and washed in sterile distilled water and bits of the mycelia placed in a nonsterile soil leachate and incubated at the desired temperature for two days. The cultures were then examined microscopically for the presence of sporangia.

Method of inoculation. Inoculum was prepared in a Waring Blendor by macerating mycelia which had been growing in pea broth for a week to 10 days. Holes were made to a depth of three inches in the soil at opposite sides of the plant to be inoculated. An aliquot of inoculum was then poured into each hole and the hole filled with soil. The check plants were treated in the same manner except that sterile pea broth was used instead of inoculum. The amount of inoculum used per plant varied from experiment to experiment, but in any one experiment the same amount of inoculum was added to each plant.

HOST RANGE AND PATHOGENICITY STUDIES

The test plants used in the host range and pathogenicity studies were 1- or 2-year-old stock from nurseries known to be free of *P. cinnamomi* and *P. lateral*is. They were planted in clay pots or greenhouse benches. When well established, they were inoculated and kept well watered. Upon the appearance of root rot symptoms the plants were removed and isolations made to recover the fungus. At the close of each experiment the roots of those plants showing no foliar symptoms were examined for possible injury.

RESULTS

HOSTS ATTACKED BY *P. CINNAMOMI* IN OREGON

P. cinnamomi has been isolated from a number of plant species in Oregon nurseries and home plantings. The fungus was isolated from two naturally infected Lawson cypress varieties, Alumi cypress, *Chamaecyparis lawsoniana* var. *allumi* (R. Smith) Beiss., and Elwoodi cypress, *C. lawsoniana* var. *elwoodi*; English yew, *Taxus baccata* L.; Irish yew, *T. baccata* var. *stricta* Laws.; Japanese yew, *T. cuspidata* Sieb. & Zucc. var. *nana* Rehd.; heath, *Erica carnea* L.; and two varieties of heather, *Calluna vulgaris* (L.) Hull. var. *alba* (West.) Don, and *C. vulgaris* var. *aurea* Don. Plants of all ages appeared to be equally susceptible.

The symptoms of the root rot of Lawson cypress varieties caused by *P. cinnamomi* are identical to those of the root rot caused by *P. lateral*is. The fungus invades the roots of the plant and spreads into the lower part of the main trunk killing all tissues as it advances. If the outer portions of the bark at the crown of a diseased plant are removed a sharp line of demarcation between living and dead cells is apparent (Fig. 1). Foliar symptoms, which involve gradual changes in color similar to these occurring when cypress trees die from transplanting injury, begin to develop at about the time the fungus reaches the crown of the plant. In the blue cypress varieties the first foliar symptom is the gradual disappearance of blue pigments until only the green undercolor remains. The color eventually fades to a tan or light brown and the foliage becomes crisp and dry. The only foliar symptom in the green varieties is a gradual fading of color until the plants are tan or light brown and dead. The first color changes are especially apparent if the plant is adjacent to a healthy plant. When the weather is cool and damp these color changes may develop over a period of several months, but if the weather is hot and dry the entire symptom sequence may occur in 2 or 3 weeks.

As the symptoms of the root rot diseases of Lawson cypress caused by *P. cinnamomi* and *P. lateral*is are identical, it was impossible to determine in the field which fungus was the causal organism. However, the two species



FIGURE 1. Root rot of Alumi cypress caused by *P. cinnamomi*. Note line of demarcation between living and dead tissue at the crown. These disease symptoms are identical to those caused by *P. lateralis*.

were readily distinguishable in the laboratory. *P. lateralis* has an optimum temperature for growth in the vicinity of 20° C. and does not grow at 30° C., whereas *P. cinnamomi* has a temperature optimum of between 25° and 30° C. and will not grow at 35° C. In addition *P. cinnamomi* has larger sporangia and grows at a much faster rate on artificial media than *P. lateralis*.

The symptoms of the root rot of English (Fig. 2), Irish, and Japanese yew are similar to those of the root rot of cypress. The fungus invades the



FIGURE 2. Root rot of English yew caused by *P. cinnamomi*. Roots of healthy (left) and diseased (right) plants.

the small feeder roots of the plant, killing them as it advances, and eventually spreads into the main roots and on up the lower portions of the stem. The line of demarcation at the crown between living and dead tissues is not as distinct as in cypress. The dead areas are usually apparent as brown vertical streaks of varying width, which extend into the wood of the stem. Foliar symptoms begin to develop when all or most of the root system has been killed. The foliage of the diseased plant fades to a tan or light brown and becomes crisp and dry. This color change may be gradual or if the weather is hot and dry may occur in a few days.

The symptoms of the root rot of the heaths (Fig. 3) and heathers differ

from those of the root rot of cypress and yew in that there is usually a localization of the foliar symptoms. The fungus invades and kills the feeder roots, slowly advances into the main roots and may advance up the lower portions of the stems. The first foliar symptoms are the fading and dying of one or more branches. This condition progresses until the entire plant is dead. Several months may be required for this sequence of foliar symptoms to develop.

The pathogenicity of *P. cinnamomi* isolates to the hosts from which



FIGURE 3. Root rot of heath caused by *P. cinnamomi*. Roots of healthy (left) and diseased (right) plants.

they had been taken was tested in the greenhouse. All of the isolates were found to be pathogenic to their respective hosts. In all cases the uninoculated check plants remained healthy.

HOST RANGE OF *P. CINNAMOMI*

Shortly after *P. cinnamomi* was found in Oregon, tests were initiated to determine the host range of an Oregon isolate of the fungus from Alumi cypress. The plants tested were selected either because they had previously been reported as hosts of the fungus or were commonly grown in Oregon nurseries. Results of the pathogenicity trials are summarized in Table I.

TABLE I
SUSCEPTIBILITY OF VARIOUS PLANT SPECIES TO AN ISOLATE OF
P. CINNAMOMI FROM ALUMI CYPRESS

Plant species	No. inoculated	No. infected
<i>Azalea mollis</i> (13)*	9	9
<i>Betula alba</i> (4, 5)	8	8
<i>Brunkenthalia spiculifolia</i>	9	9
<i>Calluna vulgaris</i> var. <i>alba</i>	9	6
<i>C. vulgaris</i> var. <i>aurea</i>	0	9
<i>Castanea dentata</i> (11)	8	8
<i>Cedrus deodara</i> (24)	6	0
<i>Chamaecyparis lawsoniana</i> var. <i>allumi</i>	6	6
<i>C. lawsoniana</i> var. <i>ehwoodi</i> (24)	6	6
<i>C. lawsoniana</i> var. <i>fletcheri</i>	6	6
<i>C. lawsoniana</i> var. <i>lutea</i>	7	7
<i>C. lawsoniana</i> var. <i>nestoides</i>	6	6
<i>C. nootkatensis</i> var. <i>compacta</i>	8	0
<i>C. obtusa</i> var. <i>crippsi</i>	7	0
<i>C. pisifera</i> var. <i>filifera</i>	7	0
<i>C. pisifera</i> var. <i>plumosa</i>	7	0
<i>C. pisifera</i> var. <i>squarrosa</i>	6	0
<i>C. thuyoides</i>	8	0
<i>Daboecia cantabrica</i> var. <i>alba</i>	9	9
<i>Daphne cneorum</i>	9	0
<i>D. odora</i>	9	0
<i>Erica arborea</i> var. <i>alpina</i>	9	9
<i>E. carnea</i> King George variety	18	18
<i>E. ciliaris</i>	9	5
<i>E. cinerea</i>	9	9
<i>E. mediterranea</i>	9	6
<i>E. terminalis</i>	9	9
<i>E. tetralix</i>	9	9
<i>E. vagans</i>	9	6
<i>Juglans regia</i> (4, 5)	8	8
<i>Juniperus chinensis</i> var. <i>pfitzeriana</i>	6	0
<i>J. excelsa</i> var. <i>stricta</i>	6	0
<i>J. sabina</i>	6	0
<i>J. squamata</i> var. <i>meyeri</i>	6	0
<i>Picea abies</i> (<i>P. excelsa</i> Link) (4, 5)	6	0
<i>Pinus mugo</i> var. <i>mughus</i>	6	0
<i>Pseudotsuga taxifolia</i> (4, 5)	10	5
<i>Quercus borealis</i> (4, 5)	11	0
<i>Rhododendron obtusum</i> Hinodegiri variety	9	0
<i>R. ponticum</i> (21)	9	9
<i>Taxus baccata</i> var. <i>stricta</i> (<i>T. baccata</i> var. <i>fastigiata</i> Loud.) (17)	9	9
<i>T. cuspidata</i> (4, 5)	9	9
<i>Thuja occidentalis</i> var. <i>aureo-variegata</i>	6	0
<i>T. occidentalis</i> var. <i>fastigiata</i>	6	0
<i>T. occidentalis</i> var. <i>woodwardii</i>	6	0
<i>T. orientalis</i> var. <i>aurea</i>	6	0
<i>T. orientalis</i> var. <i>compacta</i>	6	0

* Numbers in parentheses indicate citation to papers in which plant was first reported as a host.

Twenty-five of the 47 plant species or varieties tested were found to be susceptible. The 3 to 10 uninoculated check plants for each species or variety tested remained free of root rot.

The five varieties of Lawson cypress tested were susceptible, although they differed in degree. Six months after inoculation neither *C. lawsoniana* var. *nestoides* nor *C. lawsoniana* var. *lutea* (R. Smith) Beiss. had been killed by the fungus but their roots were severely damaged and top growth was markedly reduced, whereas *C. lawsoniana* var. *allumi*, *C. lawsoniana* var. *elwoodi* and *C. lawsoniana* var. *fletcheri* were killed in 1 to 3 months. Other species of *Chamaecyparis* tested did not appear to be susceptible. *C. nootkatensis* (Lamb.) Spach. var. *compacta* Beiss., *C. obtusa* (Sieb. & Zucc.) Endl. var. *crippsi* (Cripps) Rehd., *C. pisifera* (Sieb. & Zucc.) Endl. var. *filifera* (Senecl.) Hartw. & Ruempl., *C. pisifera* var. *plumosa* (Carr.) Otto, *C. pisifera* var. *squarrosa* (Endl.) Beiss. & Hochst., and *C. thyoides* (L.) B.S.P. showed no root damage 4 months after inoculation.

The 12 species and varieties of 4 genera of heathers or heaths tested were found to be susceptible to the fungus. However, there was considerable variation in the degree of their susceptibility. *Calluna vulgaris* var. *aurea* was much more susceptible than *C. vulgaris* var. *alba*. Similar variation was observed in naturally infested plantings. *Brunkenthalia spiculifolia* (Salisb.) Reichenb., *Daboecia cantabrica* (L.) K. Koch var. *alba* (D. Don) Dipp., *Erica arborea* (L.) var. *alpina* Bean, *E. carnea* (King George variety), *E. cinerea* L., *E. terminalis* Salisb., and *E. tetralix* L. appeared to be more susceptible than *E. ciliaris* L., *E. mediterranea* L., and *E. vagans* L.

Other plants found to be susceptible were *Azalea mollis* Blume, *Betula alba* L., *Castanea dentata* (Marsh.) Borkh., *Juglans regia* L., *Pseudotsuga taxifolia* Brit., *Rhododendron ponticum* L., *Taxus baccata* L. var. *stricta* Laws., and *Taxus cuspidata* Sieb. & Zucc. The following plants were not susceptible: *Cedrus deodara* (Roxb.) Loud., *Daphne cneorum* L., *D. odora* Thunb., *Juniperus chinensis* L. var. *pfitzeriana* Spaeth, *J. excelsa* Bieb. var. *stricta* Gord., *J. sabina* L., *J. squamata* Lamb. var. *meyeri* Rehd., *Picea abies* (L.) Karst., *Pinus mugo* Turra var. *mughus* (Scop.) Zenari, *Quercus borealis* Michx. f., *Rhododendron obtusum* Planch. (Hinodegiri variety), *Thuja occidentalis* L. var. *aureo-variegata* Henk. & Hochst., *T. occidentalis* var. *fastigiata* Jaeg., *T. occidentalis* var. *woodwardii* Spaeth., *T. orientalis* L. var. *aurea* Senecl., and *T. orientalis* var. *compacta* Beiss.

Plants found susceptible which had not previously been reported as hosts of *P. cinnamomi* were *Brunkenthalia spiculifolia*, *Calluna vulgaris* var. *alba*, *C. vulgaris* var. *aurea*, *Chamaecyparis lawsoniana* var. *allumi*, *C. lawsoniana* var. *fletcheri*, *C. lawsoniana* var. *lutea*, *C. lawsoniana* var. *nestoides*, *Daboecia cantabrica* var. *alba*, *Erica arborea* var. *alpina*, *E. carnea* (King George variety), *E. ciliaris*, *E. cinerea*, *E. mediterranea*, *E. terminalis*, *E. tetralix*, and *E. vagans*.

PHYSIOLOGICAL SPECIALIZATION OF *P. CINNAMOMI*

Other workers have shown (6, 8, 21) that physiological strains of *P. cinnamomi* probably exist. Because the existence of a large number of physiological strains would have a bearing on control measures, an attempt was made to determine the extent of physiological specialization in the species, particularly among the Oregon isolates.

Fourteen isolates of *P. cinnamomi* from various sources were tested for differences in pathogenicity using Alumi cypress, Irish yew, English walnut (*Juglans regia*) and Douglas fir (*Pseudotsuga taxifolia*) as differential hosts. Nine rooted Alumi cypress cuttings were planted in each of fifteen 22-X22-inch sections of a greenhouse bench. Rooted Irish yew cuttings were planted in the same manner. Water movement between sections was prevented by lining the sections with heavy waterproof paper. Four 2-year Douglas fir seedlings were planted in each of seventy-two 12-inch clay pots. Two-year English walnuts were planted in a ground bed in 15 widely-spaced blocks containing 6 plants per block. When the plants were well established, the plants in each block or section of Alumi cypress, English walnut and Irish yew and each 5-pot lot of Douglas fir were inoculated with a different isolate of the fungus. Approximately 3 months after inoculation the plants were removed and examined for root rot.

Four physiological strains were differentiated among the 14 isolates of *P. cinnamomi* tested (Table II). The five Oregon isolates were among

TABLE II

PATHOGENICITY OF 14 ISOLATES OF *P. CINNAMOMI* TO FOUR PLANT SPECIES

Source of isolate		Number of diseased plants			
Host	Location	Alumi cypress (9)*	Irish yew (9)*	Douglas fir (20)*	English walnut (6)*
Avocado	California	5	7	20	6
Camellia	Alabama	0	0	18	0
Chestnut	Georgia	0	5	0	5
English yew	Oregon	5	6	19	6
Heath	Oregon	8	7	18	5
Heather	California	5	4	18	6
Heather	Oregon	6	4	20	4
Irish yew	Oregon	8	6	20	4
Japanese yew	Maryland	4	9	19	0
Japanese yew	North Carolina	6	8	17	5
Japanese yew	Oregon	9	4	17	6
Pineapple	Oahu, T.H.	4	7	18	0
Shortleaf pine	Southeastern U.S.	4	8	18	6
Yew	California	6	4	19	4
Check (no inoculum)		0	0	0	0

* Number of plants inoculated with each isolate.

the 10 which were pathogenic to all four differential hosts. The four strains can be differentiated as follows: Strain 1, pathogenic to all four hosts; Strain 2, pathogenic to Douglas fir but not to Alumi cypress, Irish yew, or English walnut; Strain 3, pathogenic to Irish yew and English walnut but not to Alumi cypress or Douglas fir; Strain 4, pathogenic to Alumi cypress, Douglas fir, and Irish yew but not to English walnut.

FACTORS INFLUENCING DISEASE DEVELOPMENT AND SPREAD

Effect of soil moisture. Observations made in the field and greenhouse indicated that a high soil moisture level favored the development of *P. cinnamomi* root rot. The effect of three soil moisture levels on the development of root rot of Alumi cypress in a heavy clay loam was studied experimentally in the greenhouse. Inverted 3-inch clay pots with a 14-mm. glass tube inserted in the drainage hole were placed in the bottom of No. 10 metal cans. The glass tubing extended about 3 inches above the edges of the cans. The cans were then filled to within an inch of the top with a weighed amount of soil. A rooted cutting of Lawson cypress was then planted in each can. When the plants were well established they were divided into 3 lots of 18 plants each. The soil moisture level in the 3 lots was brought to moisture levels of 40, 65, or 90 per cent of saturation and maintained by weighing daily and adding the required amount of water through the glass tube. Nine of the plants at each of the three moisture levels were inoculated with a culture of *P. cinnamomi* which had originally been isolated from Alumi cypress. Five weeks after inoculation all of the plants were removed and the percentage of rotted roots per plant estimated.

The average percentages of inoculated Alumi cypress roots necrosed by the fungus were 86 and 83 per cent at the 90 and 65 per cent moisture levels, respectively. Only 53 per cent of the roots were killed at the 40 per cent moisture level. The uninoculated check plants remained healthy at all moisture levels, but there was a slight reduction of growth at the low moisture level.

Effect of soil texture. The effect of soil texture on the development and spread of root rot of Lawson cypress was studied under greenhouse conditions. A greenhouse bench was divided into 3 blocks of four 22- \times 22-inch sections. A section of each of the 3 blocks was then filled with one of the following soil mixtures: heavy clay loam, 3 parts heavy clay loam to 1 part sand, 1 part heavy clay loam to 1 part sand or 1 part heavy clay loam to 3 parts sand. Three rows of 3 rooted Alumi cypress cuttings were then planted in each section. When the plants were well established one of the corner plants in each section was inoculated with an isolate of *P. cinnamomi*, which had originally been isolated from Alumi cypress.

Eleven months after inoculation, spread and development of root rot

TABLE III

EFFECT OF SOIL TEXTURE ON THE DEVELOPMENT AND SPREAD OF *P. CINNAMOMI*
ROOT ROT OF ALUMI CYPRESS

Soil mixture		No. of diseased plants in each replicate of 9 plants			Average percentage of plants diseased
Clay loam %	Sand %				
100	0	1	1	1	11
75	25	7	5	6	67
50	50	1	2	2	19
25	75	1	1	2	15

away from the inoculated plant was nil or slight in a heavy clay loam, in a 1 part heavy clay loam to 1 part sand, and in a 1 part heavy clay loam to 3 parts sand mixture (Table III). However, in the 3 parts heavy clay loam to 1 part sand mixture there was considerable spread and development of root rot with an average of 67 per cent of the plants being diseased.

Effect of temperature on sporangial production. The major spread of the root rot diseases caused by *P. cinnamomi* and *P. lateralis* in Oregon has been thought to occur during the winter months when the soil frequently becomes saturated with water. Zentmyer (22), however, believed that as *P. cinnamomi* forms sporangia only between temperatures of about 70° F. and 88° F. and as most of the infection in the field probably results from zoospores, the main infection period in California is in the late spring and early summer after the soil warms up sufficiently for sporangial formation. Using Oregon isolates of *P. cinnamomi* and *P. lateralis*, the effect of temperature on sporangial production was studied in an effort to determine the possible influence of soil temperature on spread of the root rot diseases caused by the two fungi.

The two fungi were found to differ considerably in their response to temperature in relation to sporangial production (Table IV). The 6 isolates of *P. cinnamomi* tested did not produce sporangia at 15° C. and 35° C., but produced sporangia at 20° C., 25° C., and 30° C. The optimum temperature for sporangial production appeared to be about 25° C. *P. lateralis*, on the other hand, produced sporangia only at 20° C.

Vertical distribution of P. cinnamomi in the soil. Soil fumigation has frequently been suggested as a possible method of eradication of *P. cinnamomi* from infested nursery soils. This method would be successful only if the fungus were restricted to the upper foot or less of soil as it is impossible to get adequate penetration of the soil below that depth with any of the soil fumigants available at the present time. Therefore, the vertical distribution of *P. cinnamomi* in the soil under diseased Elwoodi cypress in a planting at Salem, Oregon, was determined in August 1951. Soil samples were taken at scattered points in the planting from under 20

TABLE IV
EFFECT OF TEMPERATURE ON SPORANGIAL PRODUCTION
BY *P. CINNAMOMI* AND *P. LATERALIS*

Species	Temperature, °C.				
	15	20	25	30	35
<i>P. cinnamomi</i>	o*	+	++	+	o
<i>P. cinnamomi</i>	o	+	+	+	o
<i>P. cinnamomi</i>	o	+	++	+	o
<i>P. cinnamomi</i>	o	+	++	+	o
<i>P. cinnamomi</i>	o	+	++	+	o
<i>P. cinnamomi</i>	o	+	+	+	o
<i>P. lateralis</i>	o	+	o	o	o
<i>P. lateralis</i>	o	+	o	o	o
<i>P. lateralis</i>	o	+	o	o	o

* o=No sporangial production; +=moderate sporangial production; ++=heavy sporangial production.

Elwoodi cypress, 2 to 3 feet in height, showing root rot symptoms. They were taken at 0.3-foot intervals to a depth of 2.4 feet which was approximately the deepest point of root penetration. The percentages of samples from which *P. cinnamomi* was isolated at the different depths are given in Table V.

The fungus was more abundant in the upper 1.2 feet of soil than at the greater depths. The percentage of samples from which the fungus was isolated was highest at the 0.6- and 0.9-foot depths. Although the frequency with which *P. cinnamomi* was found gradually decreased at the greater depths the fungus occurred to a limited extent even at a depth of 2.4 feet.

TABLE V
VERTICAL DISTRIBUTION OF *P. CINNAMOMI* IN THE SOIL
UNDER DISEASED ELWOODI CYPRESS

Soil depth, feet	Incidence of <i>P. cinnamomi</i> , per cent of 20 samples
0.3	60
0.6	100
0.9	95
1.2	80
1.5	40
1.8	20
2.1	40
2.4	10

DISCUSSION AND CONCLUSIONS

On the basis of these studies and those of other workers it appears that *P. cinnamomi* is potentially a very serious plant pathogen in Oregon. The fungus attacks a wide range of ornamental plants and experimentally it was shown that five Oregon isolates were pathogenic to Douglas fir

seedlings. Although the pathogen has not been shown to damage large Douglas fir trees, this aspect is probably worthy of further study since *P. cinnamomi* has become an important plant pathogen elsewhere.

As four physiological strains were differentiated from among only 14 isolates it appears likely that more than four physiological strains of *P. cinnamomi* probably exist. If additional differential hosts had been used it is probable that the number of strains differentiated would have been increased. Mehrlich (8) and White (21) have also reported the probable existence of a number of physiological strains. Zentmyer (23), however, states that he has found no differences in pathogenicity among isolates of *P. cinnamomi* from different hosts.

Several rather incongruous results obtained in the present pathogenicity studies can perhaps be explained as due to the existence of physiological strains. The isolate of *P. cinnamomi* used was not pathogenic to *Quercus borealis*, *Picea abies*, and *Cedrus deodara* which have been reported (5, 24) as being susceptible, but was pathogenic to *Erica tetralix* and *E. cinerea* which have been reported (12) as not being susceptible.

Experimentally it was found that high soil moisture favored disease development. This substantiates observations made in the field and greenhouse. In nurseries the disease was usually observed in areas that were poorly drained or kept well irrigated. Other workers have also reported (5, 21, 25) that root rots caused by *P. cinnamomi* developed more rapidly and severely at high soil moisture levels.

Spread of the disease was much greater in a soil of moderate texture than in soils of a heavier or lighter texture. Zoospores may not be able to move freely in soils of heavy texture with the result that spread of the disease is not great. Spread is restricted also in soils of very light texture because of the rapidity with which the water necessary for zoospore dissemination drains away. A soil of moderate texture is apparently optimum for conditions of proper moisture and ease of movement necessary for zoospores to move and infect roots of susceptible plants.

In Oregon most of the infection of susceptible plants by *P. cinnamomi* probably does not take place until late spring or early summer when the soil has warmed up sufficiently for sporangial production. *P. cinnamomi* was found to produce sporangia only in a temperature range of about 20° C. to 30° C. *P. lateralis* produced sporangia only at 20° C., indicating that zoospore infections can occur only within a very restricted range of soil temperature. Since zoospores are probably the primary agents of infection of both species of *Phytophthora*, it is unlikely that much infection occurs during the winter when soil temperatures are below the minimum for sporangial production.

In the soil under diseased Elwoodi cypress, *P. cinnamomi* was found to a depth of 2.4 feet but was most abundant in the upper 1.2 feet of soil.

Campbell (3) found that *P. cinnamomi* was most abundant at 2- and 3-inch depths but occurred to a limited extent at a depth of 12 inches in infested shortleaf pine stands. Anderson (1) reported that in Hawaii this fungus had been recovered to a depth of 27 inches in very compact subsoil. These differences in the vertical distribution of *P. cinnamomi* in the soil are probably due to differences in moisture content and texture of the soil and the depths to which roots of host plants have penetrated. Because of the depths at which *P. cinnamomi* may occur in the soil, chemical eradication of the fungus does not appear to be feasible.

SUMMARY

P. cinnamomi was found to be the cause of root rot of two Lawson cypress varieties, Alumi cypress, *Chamaecyparis lawsoniana* var. *allumi*, and Elwoodi cypress, *C. lawsoniana* var. *elwoodi*; English yew, *Taxus baccata*; Irish yew, *T. baccata* var. *stricta*; Japanese yew, *T. cuspidata*; heath, *Erica carnea*; and two varieties of heather, *Calluna vulgaris* var. *alba* and *C. vulgaris* var. *aurea* in Oregon nurseries and home plantings. Twenty-five of 47 plant species or varieties tested, including those from which the fungus was isolated in Oregon, were susceptible to an isolate of *Phytophthora cinnamomi*. Sixteen of the plants found to be susceptible had not previously been reported as hosts of the pathogen. Four physiological strains were differentiated among 14 isolates of *P. cinnamomi* tested using Alumi cypress, English walnut, Irish yew and Douglas fir as differential hosts.

Soil of a moderate texture appeared to be more favorable for the development and spread of root rot caused by *P. cinnamomi* than lighter or heavier soils. Disease development was also found to be favored by a high soil moisture level. The period when most infection of susceptible plants by *P. cinnamomi* and *P. lateralis* occurs is probably in late spring or early summer after the soil warms up sufficiently for zoospore production by the pathogen, rather than during the winter months. As *P. cinnamomi* may occur in the soil to a depth of at least 2.4 feet, fumigation does not appear to be a practical method for eradicating the fungus from infested soil.

The ease with which *P. cinnamomi* is disseminated, the wide host range of the fungus, and the lack of any practical means of controlling the diseases caused by this pathogen may result in *P. cinnamomi* becoming a very serious plant pathogen in Oregon.

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Notes

PERIODICAL CICADA

ALBERT HARTZELL

The appearance of the periodical cicada, *Magicicada septendecim* (L.), (often erroneously called the seventeen-year locust) during May and June of 1953, in certain sections of Eastern United States, stimulated interest in records of a previous occurrence of this insect in 1928. As is well known, this species of cicada is remarkable in its adolescent period because of its subterranean habit. The life cycle is believed to be dependent on climatic factors since in the southern part of this country the seventeen-year race is replaced by a thirteen-year race.

The periodical cicada has several geographical broods (1). These have been numbered I through XXII. Several broods are now believed to be extinct. The 1953 occurrence was of Brood X, while that of 1928 was of Brood XII.

The recent appearance of the periodical cicada in Eastern United States induced the author to delve into some hitherto unpublished notes on the habits and life history of this species. In 1928, the adults were first observed on June 7, in Hastings-on-Hudson, N. Y. A number of newly emerged adults were found congregating on some peony plants (Fig. 1 G) adjacent to an apple tree on the bark of which were a number of exuviae or cast "skins" from which the adults had emerged (Fig. 1 F). Various stages of the molting process were observed and photographed on June 14, 1928 (Fig. 1 B and C).

Since little was known of the subterranean habits of the cicada, an attempt was made to rear the nymphs of this species of insect on tree roots in the greenhouse so that the individuals could be examined from time to time and photographed at various growth stages. Maintaining the nymphs on roots of trees kept constantly under greenhouse conditions might give some information concerning the effect of temperature on the length of the life cycle of this species. Individuals of the seventeen-year race possibly might develop into adults in thirteen years if grown continuously under greenhouse conditions.

On August 28, 1928, cicada nymphs were observed hatching and emerging from egg scars (Fig. 1 A) on oak twigs collected locally by Dr. Irene D. Dobrosky. Upon hatching, the nymphs fell from the twigs and began burrowing into the soil. Newly hatched nymphs (Fig. 1 E) averaged 2 mm. in length.

Oak twigs containing egg scars were placed near a red maple seedling growing in a pot in the greenhouse so that the cicadas on hatching could enter the soil and feed on its roots. On August 5, 1931, approximately

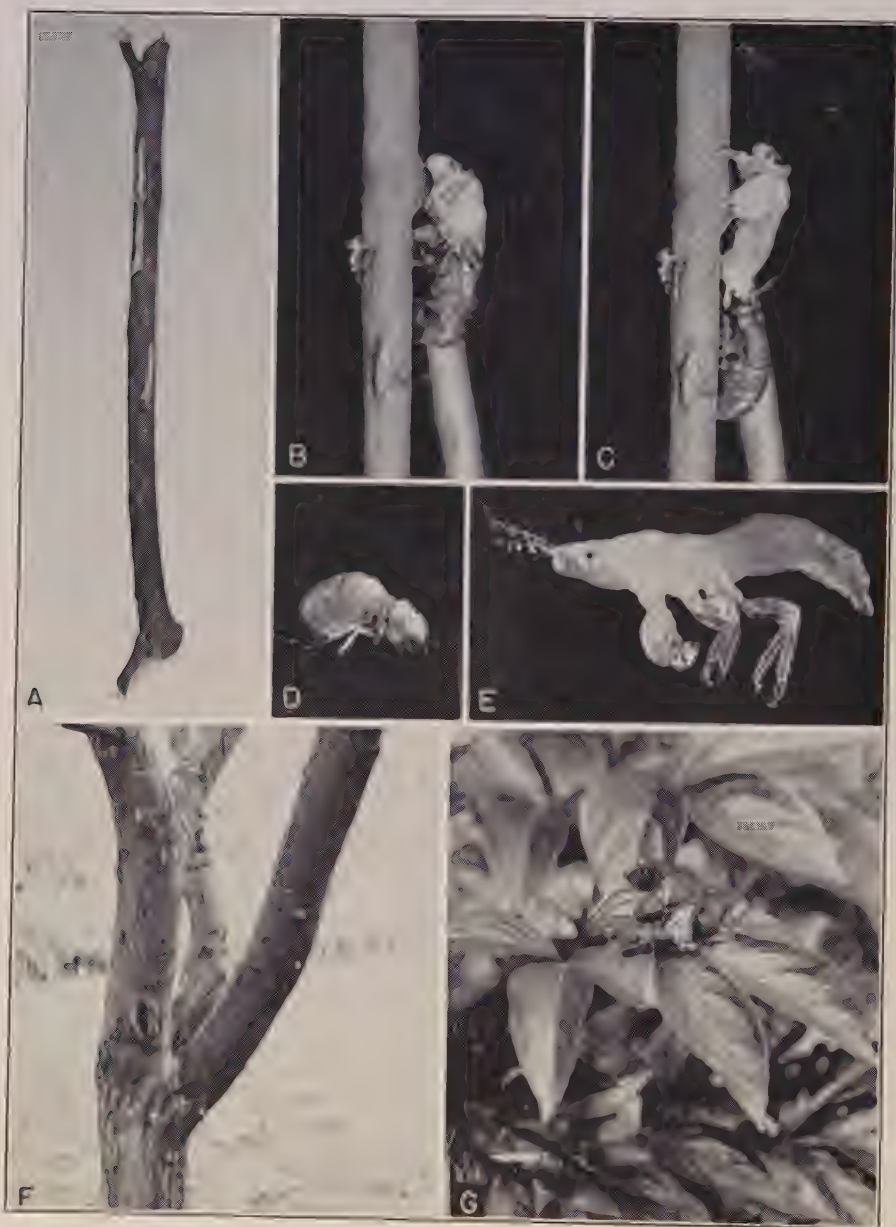


FIGURE 1. Periodical cicada, *Magicicada septendecim* (L.). A. Egg punctures on oak twig. B and C. Two stages in the molting of cicada. D. Cicada nymph aged approximately 2 years and 10.5 months, reared in captivity from an egg. E. First instar nymph (greatly enlarged), newly hatched. F. Cast "skins" on the bark of an apple tree. G. Newly emerged adults resting on peony leaves.

two years later, the roots were examined and a number of living cicada nymphs found. One individual that measured 10 mm. in length was photographed (Fig. 1 D) alive after which all were returned to the roots of the maple seedling and the tree repotted. These nymphs were approximately 2 years and 10.5 months old at the time this examination was made.

Another examination of the roots of the maple tree was made on June 24, 1932. Only one live cicada nymph was found. This nymph measured 15 mm. in length. The age of this nymph was approximately 3 years and 9 months.

The third examination of the roots of the maple tree was made on August 31, 1933. A live nymph 14 mm. in length was found, indicating that various growth stages were present in the population at the same time or that the growth of certain individuals was greatly retarded.

The fourth examination of the roots of the maple tree was made on July 10, 1935, approximately seven years after the experiment was begun. A live cicada answering to the description (2) of a fourth instar nymph was found which measured 15 mm. in length. This individual together with the soil was returned to the roots of the maple tree and the tree repotted. The tree was kept in a cage in the greenhouse to prevent the escape of adults, should any emerge.

A final examination of the roots of this maple tree was made on June 11, 1938, but unfortunately no cicadas were found. The sifted soil from the pot in which the tree had grown also failed to yield any living cicadas.

The greenhouse in which this experiment was conducted was kept at approximately 70° F. during the greater part of each year. During the summers the greenhouse was maintained without artificial heat.

While the data presented here are too meager to permit any sweeping conclusions, it is of interest to note that under greenhouse conditions periodical cicada nymphs made a growth in length of 10 to 15 mm. in approximately three years. Under field conditions Marlatt (2) has reported that it requires about eight years for nymphs to attain this length.

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NOTES ON THE LEAFHOPPERS OF BERMUDA

ALBERT HARTZELL

A brief visit to Bermuda during the fall of 1929 afforded the writer an opportunity to collect a number of species of leafhoppers (Cicadellidae). The following notes are based mainly on collections made at that time. Previous records have been made by Jones, Uhler, Van Duzee, and Ogilvie. Ogilvie (2) has published a check list of insects of Bermuda enumerating all the previous records, which has proved a valuable guide to the more common species. Dr. Herbert Osborn (4) has also collected in Bermuda but as yet no list of his collection has appeared.

Oceanic islands, such as Bermuda, have never had land connections with continents and, therefore, possess a more restricted fauna than do continental islands, which at some time in the geological past have been part of the neighboring continent. On the other hand, the fauna of oceanic islands is not strictly comparable with that of archipelagos such as the Greater Antilles, the islands of which are remnants of a former continent. The latter possess relics in addition to species brought in by recent migrations, and are consequently much richer in species than are oceanic islands. All the animal inhabitants of oceanic islands, therefore, have reached their destination by crossing the ocean, either by means of flight or drift, or through the agency of man or other animals. An insular environment tends to become more intensified as the size of the island decreases, chiefly due to the fact that the climate of a very small low-lying island group, such as Bermuda, is regulated to a greater extent by ocean currents than by atmospheric factors; and the high humidity and the presence of strong winds tend to make ecological conditions unfavorable for insects, especially for those species that possess wings.

The terrestrial fauna is believed by Verrill (7) to have reached Bermuda since the latter Pleistocene and is very sparse even for oceanic islands. In a list of 225 species of insects he reported 90 per cent as common to Bermuda and to the United States. Very few species of land animals have been reported. The peculiar conditions of isolation, low elevation and small size have tended to give Bermuda an extremely restricted fauna. The presence of strong winds has reduced the number of flying insects while its maritime climate gives the species a tendency toward darker coloration or melanism. Ogilvie lists 494 species of insects from Bermuda. One is struck by the absence of certain groups of Homoptera. There is a single species of cicada (*Cicada bermudiana* Verrill), and that endemic to Bermuda, and no species of Cercopidae or Membracidae are listed. The leafhopper fauna of Bermuda compares favorably in number of species with that of other small islands. Heidemann and Osborn (1) reported 13 species of leafhoppers from the Isle of Pines and Uhler (5) reported 19 species from the island of St.

Vincent. It appears that neither the Isle of Pines nor the island of St. Vincent is relatively richer in leafhopper species than Bermuda from which a total of 21 species and varieties are known. Both islands are somewhat larger than Bermuda and are near extensive land masses rich in homopterous species.

The writer wishes to express his sincere appreciation to Mr. E. A. McCallan, Dr. H. S. Cunningham and Mr. J. C. Nauen, formerly with the Bermuda Department of Agriculture, for kindly accompanying him to localities favorable for collecting insects and for identifying plants on which leafhoppers were collected. Special credit also is due to Dr. Herbert Osborn of Ohio State University, for determining several species and verifying others in the accompanying list of leafhoppers. In this brief listing no attempt is made at a taxonomic review of the Bermuda Cicadellidae. The present list follows, in the main, the classification of Van Duzee (6), the generally accepted guide of the Nearctic fauna at the time this collection was made. The reader is referred to Oman's (3) publication on Nearctic leafhoppers for present views on classification.

LEAFHOPPERS (CICADELLIDAE) COLLECTED IN BERMUDA

Agallia sanguinolenta (Prov.). A single specimen taken from *Oxalis* near Agric. Station, Oct. 21, 1929.

Draeculacephala mollipes (Say). Specimens taken from *Oxalis* near Agric. Station, Oct. 21, 1929.

D. minor (Walk.). A single specimen. Same place and date as above. This form has not hitherto been reported from Bermuda.

Deltocephalus flavicosta (Stål). Two light-colored specimens collected near a fiddle wood (*Citharexylum spinosum* L.) thicket about one-half mile west of Agric. Station, Oct. 24, 1929.

Euscelis exitiosus (Uhl.). Collected from grass plot near Agric. Station, Oct. 21, 1929.

Thamnotettix nigrifrons (Forbes). Most abundant species collected. Near Agric. Station, Oct. 21, 24, 1929.

T. colonus (Uhl.). Collected near Agric. Station, Oct. 24, 1929.

Cicadula punctifrons (Fall.) var. *repleta* Fieb. Collected near Agric. Station, Oct. 21, 1929. Not hitherto reported from Bermuda.

C. sexnotata (Fall.). Collected near Agric. Station, Oct. 21, 1929.

Eugnathodus abdominalis (Van D.). Found abundantly in bog between Agric. Station and Hungry Bay, Oct. 21, and in the vicinity of Grape Bay, Oct. 24, 1929. New record for Bermuda.

Dikraneura abnormis (Walsh). Collected Oct. 21, 1929 at margin of Mangrove Bay where they were observed feeding on *Paspalum vaginatum* Sw. and St. Augustine grass (*Stenotaphrum secundatum* [Walt.] Kuntze) at Hungry Bay. New record for Bermuda.

- Empoasca fabae* (Harr.). Collected from potato (*Solanum tuberosum* L.) at Agric. Station, Oct. 21, 1929.
- E. flavescens* (Fabr.). Collected from vegetation in bog situated between Agric. Station and Hungry Bay, Oct. 21, 1929. This species not hitherto reported from Bermuda.
- E. minuenda* (Ball). Collected from avocado (*Persea americana* Mill.) at Agric. Station, Oct. 24, 1929.
- E. minuenda* var. *moznettei* (Ball). Collected from avocado at Agric. Station, Oct. 24, 1929. Not hitherto reported from Bermuda.
- E. minuenda* var. *clavigerana* (Ball). Collected from avocado at Agric. Station, Oct. 24, 1929. New record for Bermuda.

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QUANTITATIVE AMINO ACID COMPOSITION OF THE
GERMAN COCKROACH, *BLATTELLA GERMANICA* (L.)

JOHN D. HILCHEY AND RICHARD J. BLOCK¹

While many qualitative determinations of the amino acids present in insects have been carried out (cited by Auclair and Dubreuil, 2) few efforts have been made to obtain quantitative data upon the occurrence of these biologically important compounds (1, 2, 4, 5). Of these, only the studies of Auclair and Dubreuil (1, 2) could be considered comprehensive. These authors published a summary of the ranges of concentrations of free amino acids present in the blood of nine species of insects studied semiquantita-

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tively (2). They have also published (1) a detailed description of the quantitative distribution of free amino acids in the blood of *Galleria mellonella* (L.).

In the course of studies of the sulfur metabolism² of the German cockroach, *Blattella germanica* (L.), it was necessary to obtain information concerning the total amounts of cystine and methionine present in samples of whole roach body solids prepared for radiological assay. The determinations were made following the "maximum color density" and "total spot area" (methionine) techniques of quantitative paper partition chromatography described by Block, LeStrange, and Zweig (3). As a corollary to this work it was possible to estimate the amounts of other

TABLE I^{male}
AMINO ACID COMPOSITION OF THE GERMAN COCKROACH,
BLATTELLA GERMANICA (L.)

Amino Acid	% Composition of dried roach	Amino acid	% Composition of dried roach
Arginine	3.0	Leucine	3.5
Histidine	0.9	Isoleucine	2.4
Lysine	1.9	Valine	2.7
Tyrosine	2.0	Glycine	3.0
Tryptophan	0.5	Alanine	4.6
Phenylalanine	3.3		
Cystine	2.2	Glutamic acid	7.5
Methionine	3.3	Aspartic acid	6.8
Serine	2.3	Proline	3.0
Threonine	3.0	Hydroxyproline	0.0?

amino acids present in these samples by the same methods. The roaches used for these tests had been fed solutions containing Na_2SO_4 labeled with S^{35} . When the insects were sacrificed for assay, the head and attached digestive tract of each insect were removed to eliminate interferences due to food residues remaining in the gut. The animals were ground and extracted with acetone, extracted with ethyl ether, and dried. Each batch of test material consisted of the water-free, fat-free body solids derived from 20 to 25 decapitated, gutted German roaches. Samples of known weight were hydrolyzed in 6 *N* HCl for 15 to 16 hours. The resulting hydrolysate was then prepared and chromatographed by means of the methods referred to above.

The results of the analyses are given in Table I.

The significance of these data lies in their usefulness in the fields of

² Unpublished data presented as a scientific paper at the Entomological Society of America Annual Meeting, Philadelphia, Pa., December 18, 1952.

insect physiology and biochemistry. Although the values found are subject to the limitations of the methods and are derived from test animals reared and studied under specialized conditions, they will perform a two-fold function. They will serve as a basis for further research into the quantitative distribution of amino nitrogen in the German cockroach and as a guide for studies of the intermediary metabolism of this and other insect species.

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COMPARISON OF CHEMICAL AND BIOASSAY METHODS FOR THE DETERMINATION OF TRACES OF CHLORDANE AND HEPTACHLOR IN FOOD CROPS¹

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INTRODUCTION

Small amounts of chlordane and heptachlor can be determined by colorimetric methods (4, 6) or through their ability to inhibit the photomigration of larvae of *Aedes aegypti* (L.) (1, 2). During a program designed to measure the accumulation of these materials in food products, a large number of samples were analyzed by both methods to determine if the results were comparable.

Samples from a series of insecticide-treated plots were extracted with *n*-pentane and the solutions chromatographed to remove interfering plant products. Analyses were made on identical aliquots to eliminate variations arising from the extraction procedure. Since the insecticide content was usually between 0.001 and 0.1 p.p.m., the results indicate the utility of the methods in their extreme range of sensitivity.

Agreement between the two methods was found to vary with the nature of the crop and details in the method of sample purification. Hence additional studies were made on the effects of various interferences on the bioassay test and on methods for eliminating them.

The data from these studies illustrate the present status of the mosquito larvae method when applied to the analysis of microgram quantities of insecticides in plant extracts, and show what further improvements would be desirable in the removal of interferences during sample preparation.

MATERIALS AND METHODS

SOURCE OF SAMPLES

Commercial formulations of technical chlordane [1,2,4,5,6,7,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene and related materials], hereinafter referred to as chlordane, and technical heptachlor [1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene], hereinafter referred to as heptachlor, were applied to field crops by cooperating growers in various sections of the United States. Dusts and sprays were used at recommended rates for the treatment of seed, soil, and foliage throughout the 1952 season.

¹ This work was supported in part by a grant from the Velsicol Corporation, Chicago, Illinois.

² The authors wish to thank Eugene P. Ordas and other members of the Velsicol staff for supplying crop extracts for bioassay, and for making available the results of their chemical analyses.

Crops on which both chemical and bioassay results were obtained include commercial varieties of potato (*Solanum tuberosum* L.), yams (*Ipomoea batatas* Lam.), cabbage (*Brassica oleracea* var. *capitata* L.), turnip (*Brassica rapa* L.), onion (*Allium cepa* L.), corn (*Zea mays* L.), and carrot (*Daucus carota* L. var. *sativa* DC.).

After harvesting, samples of the produce were shipped to the laboratories of the Velsicol Corporation for extraction and chemical analysis. Aliquots of the extracts were then made up in acetone solution and sent to this institute for bioassay. The time elapsing between chemical and bioassay tests varied from two to six weeks.

EXTRACTION AND CHEMICAL ANALYSIS

Samples for chlordane analysis were macerated, mixed with an equal volume of anhydrous sodium sulfate, and extracted in a Soxhlet apparatus with *n*-pentane for four hours. The extracts were concentrated to a volume of 10 ml. and chromatographed on Florisil columns (60 to 100 mesh) to remove plant pigments. The columns were then washed with *n*-pentane, and the percolates and washings concentrated to 5 to 10 ml. Following this, the concentrated solutions were passed through columns packed with chromatographic grade alumina which had previously been activated by drying for three hours at 120° C. under a pressure of 20 mm. of mercury. The alumina adsorbed the chlordane, and the lipids passed through the columns with the percolates. The columns were then eluted with ethyl ether, and the eluates evaporated to dryness at 40° C. under a current of air. The amount of chlordane was then determined by the colorimetric method described by Davidow (4).

Samples containing heptachlor were extracted with pentane and chromatographed on Florisil columns in a similar manner except that the clean-up technique did not include separation of the insecticide from lipids on alumina. The heptachlor in the extracts was determined colorimetrically by the method of Polen and Silverman (6).

BIOASSAY PROCEDURE

The pentane solutions containing insecticides and residual plant products were evaporated under a current of air at 40° C. and redissolved in acetone at a rate of 1 kg. of crop sample (wet weight) per ml. One-tenth to 0.5 ml. of the acetone concentrate from each sample was diluted with 100 ml. of water containing 1.0 p.p.m. Pluronic F-68 (2) and 100 larvae of *Aedes aegypti* (L.). The time for inhibition of 50 per cent of the larvae toward photomigration was determined by the method previously described (1). Standard curves were run each day on chlordane at concentrations from 0.1 to 0.01 p.p.m. and on heptachlor at 0.1 to 0.002 p.p.m. The concentration of insecticide in the unknowns was determined by inter-

polation on these curves, and the results converted to parts per million on the wet weight of the crop by the following relationship:

$$\text{p.p.m. on sample} = \frac{100 P}{Gv}$$

where P is the parts per million in the test suspension, G is the wet weight of the crop extracted and concentrated into 1 ml. of acetone solution, and v is the volume of extract used to prepare the test suspension.

Larvae used for the photomigration test varied from day to day in age and resistance since concurrent studies were in progress on rearing methods and standard conditions had not been arrived at (2). However, results were always computed relative to standard curves determined on larvae withdrawn from the same batch.

EVALUATION OF INTERFERENCES

The effects of polyethylene glycol "400," Carbowax "1000," Pluronic F-68, Vatsol OT, and Triton X-100 in masking toxicity were determined by preparing solutions of these materials in Carbitol [2-(2-ethoxyethoxy)-ethanol] containing 10 p.p.m. heptachlor and diluting 1 ml. to 100 ml. with water for the mosquito larvae test. These materials were evaluated at concentrations ranging from 1.0 p.p.m. to 10 per cent in the presence of 0.1 p.p.m. heptachlor.

Olive oil, stearic acid, cholesterol, cetyl alcohol, and the hexane extract from processed green beans were also evaluated for masking effects at concentrations of 1.0, 10, and 100 p.p.m. in the presence of 0.1 p.p.m. heptachlor. In the case of olive oil, the effect of insecticide concentration was studied by keeping the concentration of oil at 1.0 p.p.m. and reducing the concentration of heptachlor from 1.0 to 0.005 p.p.m.

Repression of toxicity by extracts from onion and turnip used at rates of 10 g. and 100 g. of the wet weight of the crop per 100 ml. of test suspension was determined at heptachlor concentrations ranging from 1.0 to 0.005 p.p.m.

Partial separations of chlordane, heptachlor, methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane], lindane [γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane], aldrin [1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene], and parathion [*O,O*-diethyl *O-p*-nitrophenyl thiophosphate] from olive oil and plant lipids were obtained by steam distillation.

In one of the methods used, the insecticide and interfering lipid were suspended in 25 ml. of a 0.1 per cent solution of Pluronic F-68 contained in a 50-ml. distillation flask. A carborundum chip coated with Dow Anti-foam A was added and the flask attached to a 25-mm. steam trap and a 200-mm. condenser equipped with standard taper joints. Heat was sup-

plied with a GlasCol mantle and the distillate was collected in a 100-ml. graduated cylinder. After *ca.* 20 ml. of water had come over, distillation was interrupted and the suspension was diluted to 100 ml. for bioassay.

Better recovery was obtained when the distillation was carried out under a current of steam. The insecticide, lipid, Pluronic F-68, and Anti-foam A in 25 ml. of water were placed in a 50-ml. flask equipped with a steam inlet tube. This was connected to a steam source and distillation was continued with concurrent heating of the flask until 50 ml. of distillate were collected. After the addition of larvae and dilution to 100 ml., bioassays were carried out by the procedures previously described.

EXPERIMENTAL RESULTS

COMPARISON OF CHEMICAL AND BIOASSAY RESULTS

During this investigation, analyses were made by the colorimetric and bioassay methods on 98 crop samples treated with heptachlor and 92 samples treated with chlordane. However, not all of the results could be included in this compilation. In a number of cases only maximum or minimum values were recorded and these could not be correlated on a statistical basis. In addition, some of the earlier samples contained traces of pentane which had a rapid anesthetic action on the larvae and obscured the results of the tests. After eliminating samples on which incomplete data were obtained or which were known to be subject to gross experimental error, there remained 80 samples containing heptachlor and 64 samples containing chlordane on which comparisons between the colorimetric and bioassay methods could be made. These included extracts from potato, yams, cabbage, turnip, onion, corn, and carrot which had been treated with insecticide under field conditions. The distribution of samples among crops was not uniform, since sample selection had to be based on the materials available rather than on experimental design.

TABLE I
COMPARATIVE RESULTS ON THE ANALYSIS OF VARIOUS CROPS FOR CHLORDANE
BY CHEMICAL AND BIOASSAY METHODS

Crop	No. of samples	Average p.p.m.		Standard deviation of mean		Chemical	Correlation coefficient
		Chemical	Bioassay	Chemical	Bioassay	Bioassay	
Potato	17	0.041	0.047	0.0046	0.0081	0.9	+0.438
Yams	3	0.040	0.021	0.0208	0.0062	1.9	+0.453
Cabbage	3	0.029	0.020	0.0080	0.0029	1.5	-0.185
Turnip	4	0.031	0.026	0.0052	0.0072	1.2	-0.052
Onion	13	0.020	0.017	0.0021	0.0027	1.2	+0.171
Corn	4	0.045	0.035	0.0174	0.0189	1.3	+0.695
Carrot	20	0.037	0.036	0.0035	0.0062	1.0	-0.084
Average	—	0.0345	0.0329	0.0055	0.0067	1.05	+0.178

The *average* values obtained by the colorimetric and bioassay methods for the analysis of chlordane on the various crops agreed closely (Table I). On potato the bioassay results were higher, while on the rest of the crops they were somewhat lower. The most serious discrepancy occurred in the case of yams where the ratio of chemical analysis to bioassay was 1.9; however, only three samples were available so the result had little significance. The over-all ratio on the 64 samples on which definitive results were obtained was 1.05, so evidently the sample preparation was adequate for the analysis of chlordane by either method.

The agreement of results within groups was less satisfactory. Correlation coefficients obtained on the various crops were between -0.185 and $+0.695$, while the average coefficient for all the chlordane-treated samples was $+0.178$. Thus the two methods gave results which agreed in order of magnitude, but failed to provide sharp distinctions within groups. Actually, the samples examined covered a comparatively narrow range since the highest chlordane content found by chemical analysis was 0.08 p.p.m., and the lowest 0.005 p.p.m. As indicated by standard deviations from the means (Table I), the ranges within groups were low.

TABLE II
COMPARATIVE RESULTS ON THE ANALYSIS OF VARIOUS CROPS FOR HEPTACHLOR
BY CHEMICAL AND BIOASSAY METHODS

Crop	No. of samples	Average p.p.m.		Standard deviation of mean		Chemical	Correlation coefficient
		Chemical	Bioassay	Chemical	Bioassay	Bioassay	
Potato	17	0.021	0.008	0.0026	0.0020	2.6	$+0.546$
Yams	5	0.026	0.003	0.0059	0.0003	8.7	-0.053
Cabbage	1	0.012	0.010	—	—	1.2	—
Turnip	19	0.022	0.006	0.0029	0.0017	3.7	$+0.169$
Onion	27	0.008	0.006	0.0012	0.0007	1.3	-0.005
Corn	5	0.016	0.010	0.0106	0.0085	1.6	$+0.906$
Carrot	6	0.030	0.008	0.0081	0.0030	3.8	$+0.549$
Average	—	0.0174	0.0067	0.0033	0.0019	2.60	$+0.252$

The average values obtained on crops containing heptachlor (Table II) differed more widely. The best agreement was obtained on onion where the ratio of chemical analysis to bioassay was 1.3, and the poorest was on yams where the ratio was 8.7. This was expected since for the most part the onion extracts gave clear solutions on dilution with water while the rest gave cloudy suspensions indicating the presence of masking agents. Comparative analyses made on 80 samples distributed among the seven crops indicated that the chemical method gave results which on the average were 2.6 times higher than the bioassay results. Evidently the inclusion of a procedure to remove lipids in the preparation of the samples containing chlordane led to better agreement between the two methods. However, the

correlation of results within groups was slightly better for the heptachlor samples since the coefficient varied from -0.053 to $+0.906$ with an average value of $+0.252$.

The average weighted correlation coefficient on the seven crops including both heptachlor and chlordane analyses was $+0.219$. There were

TABLE III
INDIVIDUAL RESULTS OBTAINED ON THE ANALYSIS OF ONION FOR HEPTACHLOR

P.p.m. heptachlor			
Bioassay	Chemical	Bioassay	Chemical
0.001	0.002	0.006	0.006
0.001	0.004	0.006	0.006
0.001	0.028	0.006	0.006
0.002	0.000	0.006	0.007
0.002	0.008	0.006	0.008
0.002	0.017	0.007	0.005
0.003	0.005	0.008	0.003
0.003	0.006	0.008	0.005
0.003	0.008	0.010	0.006
0.003	0.022	0.010	0.010
0.004	0.003	0.011	0.011
0.005	0.000	0.011	0.012
0.006	0.003	0.017	0.012
0.006	0.005		

in all 143 samples with 115 degrees of freedom, so the correlation is significant at the 5 per cent level and just short of significance at the 1 per cent level.

In several cases the agreement between the methods appeared to be better than indicated by the correlation coefficients. For example the individual results on onion (Table III) agreed satisfactorily with the exception of three cases: the third, sixth, and tenth items shown in the table. The remaining 24 pairs of results were as close as could be expected considering the small amounts of heptachlor and the reproducibility of the methods. Including all of the results, the correlation coefficient was -0.005 but when the three pairs mentioned were excluded from the calculations it was increased to $+0.643$. Since there were no obvious errors made in these determinations it would be inadvisable to omit them. The data seem to indicate that correlation was considerably reduced by occasional results that were outside the normal limits of error. It is possible that these were caused by the presence of interfering materials incompletely removed during sample preparation rather than by failure of the analytical techniques.

Not all of the groups showed this trend. For example, the average values obtained for the analysis of chlordane in carrot (Table I) agreed very well although the correlation coefficient was -0.084 . On inspecting

the individual results it was evident that the low correlation was caused by a general lack of agreement between pairs rather than the influence of a few results which differed widely from the norm. In this case the two methods agreed very well on the average amount of chlordane present, but did not distinguish between samples within the range studied.

MASKING EFFECTS BY LIPIDS

It had been shown previously (1) that the presence of lipids in plant extracts tended to mask the results of bioassay tests although the extent and variability of the masking had not been demonstrated for any great number of materials. Since this observation was part of the basis for the clean-up procedures used in the preparation of plant extracts, the effects of interferences were investigated in more detail to determine the maximum amounts that could be tolerated, and whether techniques in current use were providing samples with satisfactory purity.

Water-soluble materials such as polyethylene glycol "400," Carbowax "1000," and Pluronic F-68 did not slow insecticidal action in concentrations as high as 10 per cent (Table IV). On the other hand, surface active agents such as Vatsol OT and Triton X-100 were inhibitory at 1.0 p.p.m. although they apparently dissolved in the test medium.

TABLE IV

EFFECT OF VARIOUS WATER SOLUBLE ADDITIVES IN REPRESSING THE INACTIVATION RATE OF 0.1 P.P.M. HEPTACHLOR TO MOSQUITO LARVAE

Material tested		T ₅₀ at 0.1 p.p.m. heptachlor	
Additive	Concn., p.p.m.	With additive	Alone
Polyethylene glycol "400"	100,000	27	26
Polyethylene glycol "400"	10,000	31	26
Carbowax "1000"	10,000	34	31
Pluronic F-68	2,000	31	29
Pluronic F-68	1.0	39	38
Vatsol OT	1.0	51	38
Triton X-100	1.0	48	38

Interference from water-insoluble materials was pronounced, and increased rapidly with concentration. Thus olive oil at 0.01 to 1.0 p.p.m. did not repress the toxicity of heptachlor at 0.1 p.p.m. appreciably, but above 1.0 p.p.m. the time required for immobilization of 50 per cent of the larvae was considerably greater than for the standard (Fig. 1). In the presence of 100 p.p.m. olive oil the T₅₀ of 0.1 p.p.m. heptachlor was 100 minutes. This would correspond to a loss of 90 per cent of the activity of the insecticide which would be inadmissible in routine bioassay work.

Not all lipids interfered to this extent. Cholesterol, cetyl alcohol, stearic acid, and the crude hexane extract from green beans had no notice-

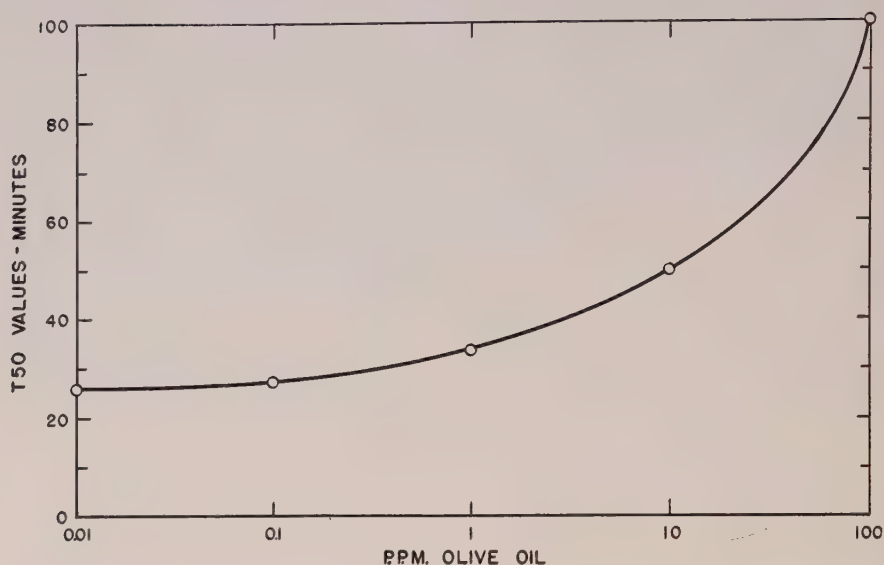


FIGURE 1. Effect of olive oil on repressing the rate of inactivation of 0.1 p.p.m. heptachlor to mosquito larvae.

able effect at 1.0 p.p.m. and repressed the rate of insecticidal action only moderately at 10 p.p.m. (Table V). At 100 p.p.m. the effect was more pronounced, although even here the decrease in apparent toxicity of 0.1 p.p.m. heptachlor was only 40 to 50 per cent compared to 90 per cent for the olive oil. Evidently fatty acids and alcohols do not inhibit as strongly as liquid glycerides. However, the effect is still serious and since plants contain fatty acids, sterols, and alcohols as well as glycerides, it is evident that the total lipid content of the test solution should be less than 10 p.p.m. to secure reliable test results.

TABLE V

EFFECT OF WATER INSOLUBLE ADDITIVES ON REPRESSING THE RESPONSE OF MOSQUITO LARVAE TO 0.1 P.P.M. HEPTACHLOR

Additive	T ₅₀ at p.p.m. added			
	0	1	10	100
Olive oil	26	33	55	100
Cholesterol	28	28	35	43
Cetyl alcohol	28	29	37	43
Stearic acid	28	28	29	37
Bean extract	28	26	31	42

The data on the crude extract from green beans are particularly interesting since this type of material would be met in practice. One hundred grams of the sample used here yielded 0.15 g. of extract. On dilution to 100

ml. with water this would correspond to 1500 p.p.m. in the test suspension, an amount greatly in excess of that which can be tolerated. However, the extract contained chlorophylls and carotenoids which also interfere. When these were removed by adsorption on Florisil the weight of the extract was reduced to 0.015 g. which would be equivalent to 150 p.p.m. total lipids in the test suspension. In this case an additional step in the purification process would be required to further reduce the lipid content.

These data illustrate the interference that would be obtained at 0.1 p.p.m. heptachlor, but do not provide information on what would happen at very low concentrations of insecticide. This situation was clarified by an experiment in which the concentration of lipid (olive oil) was maintained constant at 1.0 p.p.m. and the amount of heptachlor reduced progressively (Table VI). At 1.0 p.p.m. heptachlor masking apparently occurred, but the results are not significant since the change in concentra-

TABLE VI

EFFECT OF 1.0 P.P.M. OLIVE OIL IN REPRESSING THE RATE OF INACTIVATION OF VARIOUS CONCENTRATIONS OF HEPTACHLOR TO LARVAE OF *Aedes aegypti* (L).

Concn. of heptachlor, p.p.m.	T ₅₀		Per cent of initial activity
	Alone	With oil	
1.0	20.5	23.5	*
0.1	38.5	43.0	70
0.05	56.0	60.0	90
0.01	108.0	141.0	56
0.005	148.5	540.0	*

* Not in significant range.

tion with respect to T₅₀ is too low for accurate measurement in this region. At 0.1 and 0.05 p.p.m. the apparent activity of the heptachlor was reduced to 70 to 90 per cent of its original value, while at 0.01 p.p.m. and below, masking increased further. At very low concentrations increases in T₅₀ values are not as important as they appear superficially, since here the rate of change of T₅₀ with respect to concentration is very high. It can be concluded that the percentage reduction in apparent activity is almost independent of the absolute amount of insecticide in the range of analytical interest.

These data illustrate in a general way the role of lipids in masking toxicity. They do not provide direct information on the masking effects that would be expected on the samples containing heptachlor discussed in the preceding section. Extracts were available from turnip and onion which contained 0.002 and 0.001 p.p.m. heptachlor, respectively, as determined by the bioassay method. Aliquots representing 10 and 100 g. of each crop were fortified with heptachlor at concentrations ranging from 0.01 to 1.0 p.p.m. and T₅₀ determinations made in the usual way. In the presence of extract equivalent to 10 g. of crop, onion was noninhibitory down to

0.05 p.p.m. heptachlor while turnip was slightly inhibitory (Table VII). In the presence of extract equivalent to 100 g. of crop slight inhibition was obtained in both cases, but the results would be satisfactory for routine bioassay. At 0.01 p.p.m. the T_{50} values of the samples were lower than those of the standards owing to the additive effect of the heptachlor already present in the extracts. At high concentrations this was negligible, but at 0.01 p.p.m. it represented a considerable portion of the total insecticide present.

TABLE VII

EFFECT OF FOOD EXTRACTS AND OLIVE OIL IN REPRESSING THE RESPONSE OF MOSQUITO LARVAE TO VARIOUS CONCENTRATIONS OF HEPTACHLOR

Heptachlor added, p.p.m.	T_{50} (minutes) with additives					
	None	1.0 p.p.m. Olive oil	Extract from grams turnip*		Extract from grams onion*	
			10	100	10	100
1.0	19.2	23.5	21.5	26.0	18.0	21.5
0.1	35.8	43.0	39.5	50.0	34.0	43.0
0.05	50.0	60.0	53.5	63.0	45.0	57.5
0.01	115.0	141.0	98.0	—	90.5	97.5
0.005	160.0	540.0	—	—	—	—

* The turnip and onion samples originally contained 0.002 and 0.001 p.p.m. heptachlor respectively as determined by bioassay.

These results indicate that heptachlor can be determined in onion and turnip down to 0.05 p.p.m. with reasonable accuracy using a single chromatographic step for the removal of plant pigments. For very low concentrations or for some other crops, additional treatment may be necessary to remove residual lipids.

RECOVERY OF INSECTICIDES BY STEAM DISTILLATION

A number of procedures have been suggested for the separation of insecticides from lipids which are more or less satisfactory depending upon the nature of the insecticide, the crop, and the method of assay (3, 5). In this work steam distillation appeared particularly attractive since both heptachlor and chlordane are volatile, and the mosquito larvae bioassay can be conveniently carried out on the aqueous distillates without further extraction or concentration.

Preliminary experiments were made by suspending the insecticide in 25 ml. of water and distilling over a 20-ml. volume in semimicro equipment. In order to determine the range of applicability of the method, parathion, lindane, aldrin, and methoxychlor were included as well as heptachlor and chlordane. Recoveries ranged from 10 to 88 per cent depending upon the insecticide and sample size (Table VIII). Heptachlor and lindane were easily recovered in 10- μ g. quantities, while 20- to 50- μ g. samples were used

TABLE VIII
RECOVERY OF VARIOUS INSECTICIDES BY DISTILLATION USING
TWO DIFFERENT TECHNIQUES

Insecticide	Expected concn. in test sus- pension, p.p.m.	% Recovery by simple distillation	% Recovery in current of steam
Heptachlor	0.1	88	100
Lindane	0.1	60	100
Parathion	0.5	44	80
Chlordane	0.5	80	66
Aldrin	0.2	55	50
Methoxychlor	0.5	10	40

for some of the others. Recoveries were improved in most cases by carrying out the distillations in a current of steam and collecting 50 ml. of distillate (Table VIII). Using this method, recoveries of heptachlor and lindane were quantitative while the yield of methoxychlor was raised from 10 to 40 per cent.

In order to determine whether this method could be used to separate heptachlor from nonvolatile lipids, suspensions containing 1 to 10 μ g. of heptachlor and 10,000 μ g. of olive oil were prepared and steam distilled. These experiments were carried out in the presence of 0.1 per cent Pluronic F-68 to improve the suspendibility of the oil and allow for better contact between the water and oil phases. Since considerable foaming occurred it was necessary to add a boiling chip coated with Dow Antifoam A.

Recoveries of 50 per cent were obtained when 20 ml. of distillate were collected by ordinary distillation of an initial volume of 25 ml. However, when the operation was carried out under a current of steam with the collection of 50 ml. of distillate the recovery was increased to 90 per cent. The presence of the oil reduced the rate of take-off, but the final recovery was in the same range obtained on suspensions of pure insecticide.

Since this method appeared applicable to the recovery of heptachlor, several crop extracts on which the bioassay results were very low com-

TABLE IX
EFFECT OF STEAM DISTILLATION IN REMOVING HEPTACHLOR FROM INTERFERING
SUBSTANCES IN EXTRACTS OF VARIOUS CROPS

Crop	P.p.m. by chemical analysis	P.p.m. by bioassay		Appearance of test suspension before distillation
		Before distillation	After distillation	
Potato	0.013	0.003	0.007	Cloudy
Yams	0.030	0.000	0.002	Very cloudy
Turnip	0.040	0.000	0.007	Cloudy
Onion	0.017	0.002	0.003	Slightly cloudy
Corn	0.010	0.000	0.002	Slightly cloudy

pared to the chemical analyses were steam distilled and retested to determine if the discrepancies were due to masking by lipids. In all cases the apparent heptachlor content was increased by this procedure indicating separation from the interferences (Table IX). However, the results were still much lower than those obtained by chemical analysis, so additional factors must be involved. It is possible that the chemical analyses were too high owing to background absorption. On diluting the acetone solutions of the crop extracts to 100 ml. with water, turbid suspensions were obtained indicating the presence of plant constituents. However, with the exception of carrot, the steam distillates were clear so it was evident that materials which might cause masking in the bioassay test had been substantially removed. Since it had been shown that heptachlor could be recovered from lipids by this procedure there was no reason to doubt that the bioassay results were approximately correct.

DISCUSSION

Average results obtained by the chemical and bioassay methods for the determination of chlordane in a number of crops agreed very well, considering the small amounts of residues present. With the samples containing heptachlor agreement was less satisfactory, probably because of the presence of residual lipids which masked the action of the insecticide in the bioassay test. Over-all correlation was significant at the 5 per cent level with a coefficient of $+0.219$ and 115 degrees of freedom. Thus there was some relation between pairs of results, but it was outweighed by other factors not subject to experimental control.

The two methods therefore agree on the order of magnitude of the residues but not on individual samples within each group. This is not unexpected considering the nature of the samples and the limitations of the analytical procedures. The highest *average* residue content was 0.047 p.p.m. found on potatoes and the lowest was 0.003 p.p.m. found on yams by the bioassay method. These values are close to the limits of sensitivity of both methods even when large samples are used. Duplicate determinations made by the colorimetric methods are reported to vary by as much as 100 per cent in this range while the bioassay results may vary by 20 per cent. In view of this, a high degree of correlation cannot be expected particularly when the differences between samples are small. The standard deviations of the means for the various crops were from 10 to 85 per cent of the average values; hence differences between samples from the same crop were frequently smaller than the errors inherent in the methods of assay.

The colorimetric methods tend to give high results since impurities present in the extracts cause background absorption. On the other hand, bioassay results are generally low since lipids mask the rate of insecticidal

action. The true values are probably somewhere intermediate between the two extremes. In the case of chlordane-treated crops this difference has been largely eliminated, but in the heptachlor samples it still persists. However, the results obtained by the steam distillation method show that it is possible to work out suitable purification methods for specific cases.

These residue analyses indicate that contamination of food crops by insecticides should not be a serious problem if amounts up to 0.1 p.p.m. can be tolerated. The extracts used in these studies were obtained from field crops treated at recommended doses for insect control and this figure was exceeded in only a few cases. Ninety-two per cent of the samples contained less than 0.05 p.p.m. residue by chemical analysis and 18 per cent less than 0.01 p.p.m. It is difficult to see how such residues could represent a hazard to the consumer.

SUMMARY

Samples of potato, yams, cabbage, onion, carrot, corn, and turnip which had been treated with insecticide under field conditions were analyzed for chlordane and heptachlor by chemical and bioassay methods. The chemical analyses were made by the methods of Davidow, and Polen and Silverman, while the bioassay results were obtained by the mosquito larvae technique described by Burchfield, Hilchey and Storrs. Average results obtained by the two methods agreed closely for the samples containing chlordane, but the bioassay method gave lower results on the heptachlor samples because of the masking effect of plant lipids present in the extracts. Subsequent work showed that heptachlor can be separated from nonvolatile lipids by steam distillation.

Correlation between the two methods was significant at the 5 per cent level when the entire group of 143 samples was considered. However the correlation coefficient was low ($+0.219$) since the methods were used close to their limits of sensitivity and variations in insecticide content between samples were small.

Data are presented on the masking of bioassay results by lipids and plant extracts which illustrate the degree of purity which must be achieved by sample clean-up techniques to obtain satisfactory results.

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COMPARATIVE VOLATILITY OF THE *n*-BUTYL,
2-ETHYLHEXYL, AND 2-(2-ETHOXYETHOXY)-
PROPYL ESTERS OF 2,4-D

LAWRENCE P. MILLER, RICHARD M. WEED, AND A. E. HITCHCOCK

The esters of 2,4-dichlorophenoxyacetic acid (2,4-D) have been hazardous to use in the vicinity of sensitive crops because of their volatility. In order not to forego the obvious advantages of esters such as oil solubility and effectiveness in destroying certain types of weeds, esters of low volatility were developed and introduced for use on crops and lawns (2).

Determination of the relative volatility of various esters has been the subject of a number of investigations. Recently Hitchcock, Zimmerman and Kirkpatrick (1) have published extensive studies concerned with a biological method for determining the volatility of esters of 2,4-D and 2,4,5-T. Estimates of relative volatility were based on physiological responses of tomato plants. The term physiological volatility was suggested as descriptive of the type of effect determined. Comparisons were made of the amount of chemical required to give a certain response when applied to filter paper enclosed in a paper bag with a small potted tomato plant, and when applied directly to a leaflet of the plant. The quantity of 2,4-D ester required to induce a modification of a given magnitude was 30 to 1000 times greater for vapor as compared with direct treatment of one leaflet. With the more volatile esters the increased amounts required are presumably the result in part of the actual escape of vapor from the containers used (which were not hermetically sealed). The still greater differences with the esters of low volatility naturally follow because certain portions of the applied ester will remain on the filter paper at the end of exposure periods of 24 hours or less. Other factors influencing the results include differences in penetrability between the esters and any differences in reactivity once the esters have entered the plants.

Recently the *n*-butyl, 2-ethylhexyl, and 2-(2-ethoxyethoxy)propyl esters of 2,4-D labeled with C¹⁴ in the carboxyl position have become available. With the labeled esters it has been possible to study evaporation rates uncomplicated by biological factors. From the viewpoint of effect on adjacent crops it is important to have information as to the relative rates at which esters deposited on various surfaces in the treated fields will volatilize, enter the atmosphere, and become available for absorption by nearby plants. Evaporation from various surfaces should be similar unless different surface areas are involved or there is some reaction between the material on which the ester is deposited and the ester. The studies reported herein have been carried out for the most part in nickel-plated planchets at room temperature in a ventilated hood. The relative volatility of the *n*-butyl, 2-ethylhexyl, and 2-(2-ethoxyethoxy)propyl esters was found to

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be approximately 50, 3, and 1. Further investigations in progress are concerned with the penetrability and movement of the esters after they reach plant surfaces.

The labeled esters were obtained through the courtesy of the Carbide and Carbon Chemicals Company and were prepared by Frank G. Young and Walter J. Skraba of their Research and Development Laboratories. They had specific activities of 40.4, 33.6, and 32.0 mc. per gram for the *n*-butyl, 2-ethylhexyl, and 2-(2-ethoxyethoxy)propyl esters respectively. They were dissolved and stored in benzene to avoid effects of internal radiation on the stability of the esters.

Rates of evaporation were studied with small quantities of the esters in one-inch cupped planchets. Even the least volatile of the esters could not be counted directly with an end window type Geiger tube without a gradual increase in background count. The addition of 0.5 to 1 mg. of potassium hydroxide in 1 ml. of water to the planchets containing the esters and subsequent drying under a heat lamp served to hydrolyze the esters and form the potassium salt. For the determination of relative volatility small portions (usually 0.2 ml.) of a benzene solution of the esters were measured out into a series of nickel-plated planchets and the benzene allowed to evaporate spontaneously. The planchets were then placed in a hood which was being ventilated with a fan. At suitable time intervals, depending upon the degree of volatility of the ester being studied, one or more of the planchets were taken, potassium hydroxide added, and the amount of radioactivity compared with that at the start of the test. The test was considered to begin when the benzene containing the ester had evaporated. There was no way of preventing some of the ester from also being lost during this process, especially with the butyl ester. This had some effect on the accuracy of the determinations in that the zero time for all the planchets in a series was not exactly the same. This accounts for some of the variability in the values obtained with this ester.

The above procedure of using replicate planchets rather than determining the radioactivity of the same planchets at various time intervals had to be followed with the butyl ester since counting without some method of fixing the C^{14} could not be considered. The same methods were also employed in studying the volatility of the much less volatile 2-ethylhexyl and 2-(2-ethoxyethoxy)propyl esters. With these, a somewhat larger number of replicated planchets was required in view of the longer time periods during which evaporation took place. As the work progressed it became evident that the position of the planchets (because of differences in air currents) in the hood played a fairly important role. When this was kept in mind reproducible results could be obtained for the two esters. Somewhat smoother curves were obtained, however, when the esters in the same planchets were counted directly at various time intervals. It was then possible to alternate the positions occupied by the two esters and so

eliminate variation due to position. In general, direct counting of the esters is not recommended because of the effects in contaminating the apparatus used.

The results with the butyl ester are summarized in Figure 1. The various points represent data obtained in nine different runs. Usually the quantity of ester used was 1.21 micrograms per planchet, although in one series quantities up to 4.84 micrograms per planchet were used without

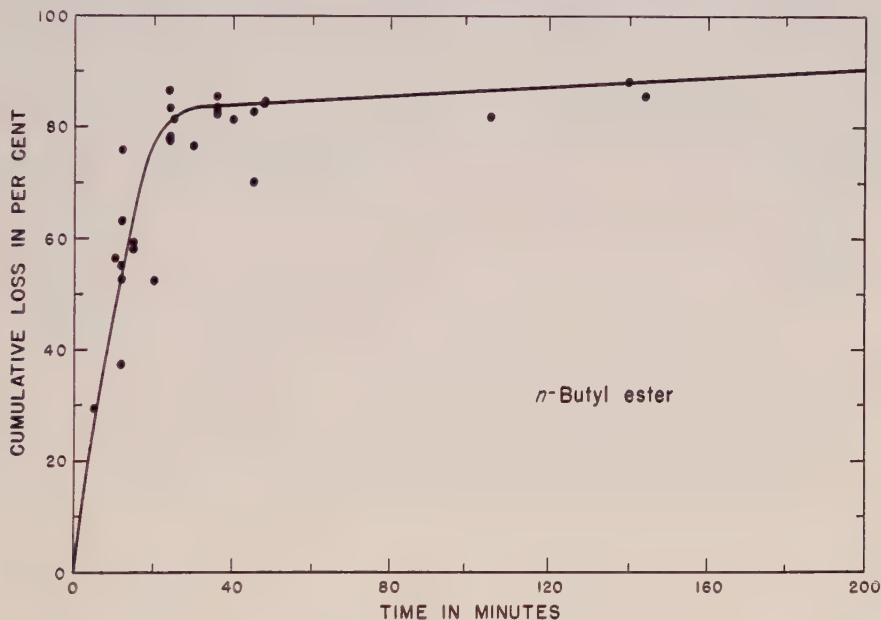


FIGURE 1. Evaporation of *n*-butyl ester plotted against time in minutes.

significant effect on the results. Similarly nickel-plated planchets were used in most instances but a run in which this surface was compared with stainless steel and glass is also included. Evaporation from the three surfaces was not significantly different. The data are all plotted on one graph and an attempt made to obtain an average value for the time required for 50 per cent loss of the ester. Reading from the curve this value is about 10.5 minutes.

The results obtained with the 2-ethylhexyl and 2-(2-ethoxyethoxy)-propyl esters are shown in Figure 2. The data presented were obtained by determining the loss in radioactivity of planchets exposed for various periods of time. Position was alternated frequently so that these showed no effect of position on the relative volatility found for the two esters. The results indicate that 50 per cent of the 2-ethylhexyl ester is lost in 2.5 hours, while 9 hours are required for the 2-(2-ethoxyethoxy)propyl ester. In other runs in which different planchets were used for each time interval,

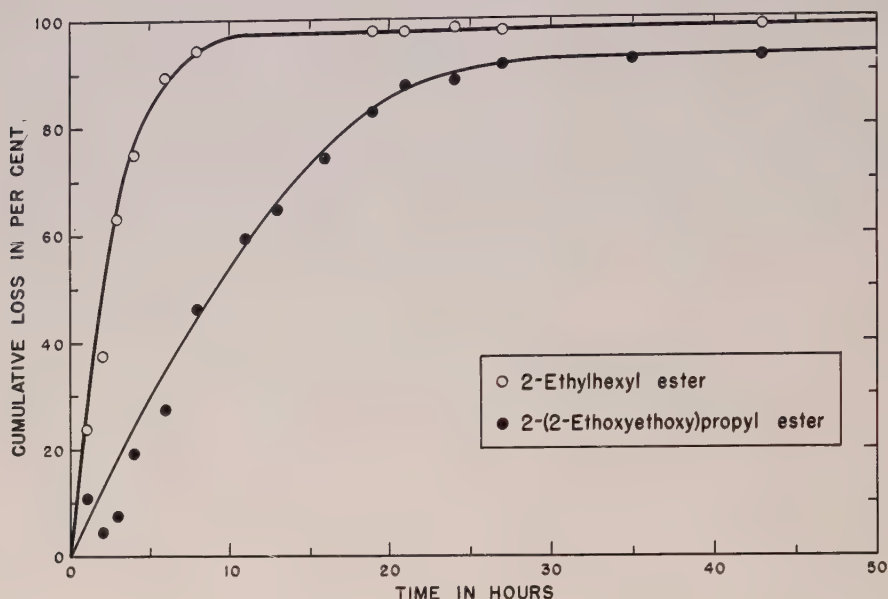


FIGURE 2. Curves showing evaporation rates of the 2-ethylhexyl and 2-(2-ethoxyethoxy)propyl esters of 2,4-D.

but in which positions in the hood giving comparable results were used, values found for the time required for 50 per cent loss were 2.5 and 3, and 8 and 8.5 for the two esters respectively.

The values obtained should be a fair measure of the relative volatility of the three esters studied. Tests carried out under different conditions would give different absolute values but the relative values should agree fairly well.

SUMMARY

The rates of evaporation of the *n*-butyl, 2-ethylhexyl, and 2-(2-ethoxyethoxy)propyl esters of 2,4-D at room temperature in a ventilated hood were determined with the aid of C^{14} -labeled esters. The esters were compared as to the time required for 50 per cent to evaporate when 1.2 to 4.8 micrograms were added to one-inch nickel-plated planchets. The time required was 10.5 minutes for the *n*-butyl ester and 2.5 and 9 hours for the 2-ethylhexyl and 2-(2-ethoxyethoxy)propyl esters respectively. The relative volatility is therefore approximately 50, 3, and 1 for the three esters.

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THE ROLE OF AUXIN IN PLANT FLOWERING. II. METHODS FOR THE EXTRACTION AND QUANTITATIVE CHEMICAL DETERMINATION OF FREE 3-INDOLEACETIC ACID AND OTHER INDOLE COMPOUNDS FROM PLANT TISSUES

A. J. VLITOS AND WERNER MEUDT

The role of 3-indoleacetic acid (IAA) and other indole compounds in the flowering processes of plants is vague. Recent evidence suggests that IAA applied to short-day plants inhibits flowering while antiauxins can reverse the inhibition (2, 3). The flowering of long-day plants grown under long-day conditions can also be inhibited by applied auxin, and long-day plants grown under short-day conditions can be induced to form floral primordia with applied IAA (2). A balance between auxin and florigen has been postulated to regulate flowering in some short-day species (3). An important adjunct to these observations and postulates would be experimental evidence that the levels of IAA in short-day plants are actually decreased during or after photoinduction and that corresponding, vegetative, short-day plants of comparable age show quantitative increases in IAA.

The bulk of the work with quantitative auxin assays has been based upon nonspecific, biological assays (i.e. *Avena* coleoptile curvature or *Avena* straight-growth tests). Therefore the precursors of IAA in plant tissues, examined with biological assays, have not been adequately considered in photoperiodic studies. However, by means of the paper chromatographic technique described in the first paper of this series (10), it is now possible to study, individually, the role in flowering of free IAA, IAA bound to proteins, and precursors which can be converted into IAA. Since some plant tissues contain inhibitors of auxin (1), the present method permits a direct study of the effect of naturally-occurring inhibitors on an auxin of known composition.

A suitable method for the extraction of free IAA and other indole compounds from plant tissue is necessary if paper chromatography is to be utilized most effectively. Although numerous methods have been published, there is no agreement on the most suitable technique, as shown in the review by Terpstra (8). She has proposed a method for extracting auxin from *Avena* coleoptiles, and from some green plant tissues, based on the water extraction of frozen material and subsequent dissociation of the extracted auxin-complex with ethyl ether. Manipulation of the pH of the extract makes it possible to separate the ether fractions into the acid and alkali fractions.

Kefford (5)¹ has described a method for the extraction of free indole

¹ The authors are indebted to Dr. Kefford for making his thesis and technique available for this work.

compounds from plant tissues employing absolute alcohol as the extracting solvent. All of the free auxin was obtained with one extraction at -12°C . The present paper is concerned with a comparison of the methods of Terpstra (8) and Kefford (5) for the extraction of free IAA and added IAA from plant tissue. The possible conversion of tryptophan to IAA or to 3-indolepyruvic acid (IPA) during the extraction was also considered using quantitative paper chromatography (10) as an analytical tool.

Several of the environmental variables which may be encountered during any extraction procedure were evaluated quantitatively for their effects upon the stability of IAA. A method is described for the extraction and quantitative determination of free IAA which precludes the formation of IAA from precursors during extraction.

EXTRACTION OF ADDED IAA AND TRYPTOPHAN FROM TOMATO TISSUE WITH WATER AND ETHER AS COMPARED WITH ABSOLUTE ETHANOL EXTRACTION

WATER-ETHER EXTRACTS

Tomato seedlings (*Lycopersicon esculentum* Mill. var. Bonny Best) were harvested by severing the stems at the soil line and immediately freezing the intact seedling by immersion into large beakers held in a mixture of dry ice and methanol. An entire seedling was frozen in six seconds. The frozen tissue was transferred to a cold room (-10°C .) and was passed once through a meat grinder. The tissue was ground to a fine powder without thawing. Fifteen lots of 20 g. each of the frozen tissue were transferred to 250-ml. beakers. The following treatments were applied to triplicate samples in the first series of tests (Series A):

Treatment 1. 80 Mg. IAA added to 20 g. of frozen, ground tissue.

Treatment 2. 80 Mg. tryptophan added to 20 g. of frozen, ground tissue.

Treatment 3. Nontreated frozen, ground tissue.

Treatment 4. 80 Mg. IAA (no tissue) dissolved in isopropyl alcohol and water.

Treatment 5. 80 Mg. tryptophan (no tissue) dissolved in water.

To each of the five treatments was added enough chilled, glass-distilled water to give a total of 100 ml. After six to eight hours each sample had frozen so that the effective diffusion of either tryptophan or IAA from treated tissue into the aqueous medium occurred only during the initial six to eight hours of extraction. Each sample was thawed 24 hours later (but kept under refrigeration), and adjusted to pH 8.0 by the addition of 50 ml. of 8 per cent NaHCO_3 . The alkaline solutions were extracted with three successive portions of cold, peroxide-free, anhydrous ethyl ether, giving a total of 150 ml. of alkaline ether extract. The addition of 30

ml. of a 1 *N* HCl solution to the residual aqueous fraction gave a pH of 4.5. Another three extractions with ethyl ether (50 ml. each) yielded the acidic ether extract. The acidic ether extracts were evaporated to dryness under reduced pressure and the residue was brought up to 10 ml. in isopropyl alcohol. All operations subsequent to the harvesting of the seedlings were performed in phototropically-inactive red light in a cold room (-10° C.).

A similar series of experiments was carried out in which there was no change to a higher pH during extraction. The water extracts were acidified directly to pH 4.5 and the acidic ether extracts were isolated (Series B).

Standard calibration curves for IAA and for tryptophan were obtained from paper chromatographs employing the maximum density of spots developed with *p*-dimethylaminobenzaldehyde (10). The amounts (in percentages) of IAA and tryptophan recovered from tomato tissue, in typical experiments, as a result of extraction with water, are given in Table I.

TABLE I

PER CENT RECOVERY OF ADDED IAA AND TRYPTOPHAN FROM CONTROL AND TREATED TOMATO TISSUE WITH WATER AT -10° C. AS DETERMINED BY PAPER CHROMATOGRAPHY

Treatment	Per cent extracted				Other indole compounds	
	IAA		Tryptophan*		A	B
	A	B	A	B		
Tissue+80 mg. IAA	67	75	0	0	0	0
Tissue+80 mg. tryptophan	0	0	80	71	6**	10**
Tissue (control)	0	0	0	0	0	0
80 Mg. IAA	62	75	0	0	0	0
80 Mg. tryptophan	0	0	85	100	0	0

* Tryptophan was recovered from the aqueous fractions.

** The Rf value of this compound corresponds to that for 3-indolepyruvic acid and agrees with that reported by Stowe and Thimann (7).

The recovery of IAA was consistently greater in Series B (pH change to 4.5 only) than in Series A where the pH was manipulated twice. In both cases the loss of IAA exceeded 20 per cent. Repeated tests have shown that as more steps are added to the extraction procedure it becomes more difficult to recover added IAA or tryptophan. The recovery of 6 per cent and 10 per cent of the added tryptophan as another indole compound, probably 3-indolepyruvic acid (IPA) is of considerable interest, since this compound was recovered only in the presence of plant tissue to which tryptophan had been added. If tryptophan was converted to IPA it occurred at temperatures (-10° C.) below which one would normally expect enzymatic reactions to occur. Under the conditions of these experiments tryptophan was not converted to IAA. The absence of IAA from control tissue samples is accounted for on the basis that 20 g. of tissue would not contain enough of the compound to be detected chromato-

graphically. The lowest limit of detection of IAA on paper chromatographs with *p*-dimethylaminobenzaldehyde as the color reagent has been estimated to be 0.1 microgram.

ABSOLUTE ETHANOL EXTRACTS

Kefford (5) has recently described a method for the extraction of free auxin from plant tissue using absolute ethanol as the solvent. Briefly, the method consists of extracting frozen, ground tissue for short periods with absolute ethanol at -12° C. The ethanol is evaporated off leaving an aqueous residue which is acidified with phosphoric acid. Glucose is added to the acidified, aqueous fraction and this is followed by extraction with ethyl ether. Application of this technique to the extraction of added IAA from tomato tissue was made in a series of experiments similar to those described for water-ether extractions. In addition to extractions of tomato tissues with absolute ethanol, mixtures of ethanol and water (4:1) were used as extraction solvents. Results of absolute ethanol and water-ethanol extractions of tomato tissues are given in Table II.

TABLE II

PER CENT RECOVERY OF ADDED IAA AND TRYPTOPHAN FROM CONTROL AND TREATED TOMATO TISSUE WITH ABSOLUTE ETHANOL AND WITH MIXTURES OF ETHANOL AND WATER AT -10° C. AS DETERMINED BY PAPER CHROMATOGRAPHY

Treatment	Per cent of IAA recovered	
	Absolute ethanol	Ethanol-water (4:1)
Tissue + 80 mg. IAA	100	100
Tissue + 80 mg. tryptophan	0	0
Tissue (control)	0	2.1*
80 Mg. IAA	100	100
80 Mg. tryptophan	0	13

* Micrograms of IAA recovered from control tissue.

The recovery of IAA from tomato tissues treated with known amounts of IAA was complete with one extraction at -10° C. with absolute ethanol, agreeing with the results of Kefford (5). However, if a mixture of ethanol and water was used as the extraction solvent, IAA was obtained from tissues treated with tryptophan as well as from nontreated tissues. These results suggest that the incorporation of water in the extraction solvent favored the conversion of tryptophan to IAA during the course of extraction. A comparison of absolute ethanol extractions (Table II) with water extraction followed by ether dissociation (Table I) indicates the suitability of the ethanol solvent for this particular tissue.

A comparison of water and ethanol extractions of spinach (*Spinacia oleracea* L. var. Nobel) tissue treated in a manner identical to that described as above for tomato tissue is recorded in Table III.

TABLE III

PER CENT RECOVERY OF ADDED IAA AND TRYPTOPHAN FROM SPINACH TISSUE WITH WATER AND WITH ABSOLUTE ETHANOL AT -10° C. AS DETERMINED BY PAPER CHROMATOGRAPHY

Treatment	Per cent recovered with			
	Water	Ethanol IAA	Water	Ethanol Tryptophan
Tissue + 80 mg. IAA	10	90	0	0
Tissue + 80 mg. tryptophan			10	85
Tissue (control)	0	0	0	0
80 Mg. IAA	25	80	0	0
80 Mg. tryptophan	0	0	40	95

As in tomato tissue, IAA and tryptophan were recovered from spinach tissue more effectively with absolute ethanol than with water. There was no detectable conversion of tryptophan to IAA during either of the extraction procedures. Repeated extractions of the tissues at -10° C. yielded negative tests for IAA or other indole compounds.

EFFECT OF ENVIRONMENTAL VARIABLES ENCOUNTERED DURING EXTRACTION ON THE STABILITY OF IAA

In addition to avoiding the conversion of precursors to IAA during extraction, it is desirable to assay the influence of environmental variables which might affect the recovery of free IAA from plant tissues. The effects of pH, light, and temperature on the stability of IAA were evaluated only insofar as they might influence the recovery of IAA for the short periods of time encountered during extraction.

EFFECT OF HYDROGEN-ION CONCENTRATION

A series of buffers was prepared ranging in pH from 1.5 to 9.0. IAA (10 micrograms per 2.5 microliters) was incubated in each buffer for a period of one, three, and seventy-six hours. Paper chromatographs were prepared after each incubation period had expired. Theoretically 10 micrograms of IAA per 2.5 microliters of buffer solution should have been recoverable. The amounts actually found to be present after each incubation period in replicated experiments are shown in Table IV.

Unless an extraction of free IAA from plant tissues is completed within a few hours there is a possibility of inactivation of the compound at low pH values. Since an acidified extract is usually not kept for more than a few hours, inactivation of IAA during extraction as a result of pH changes would not be expected.

EFFECT OF LIGHT

The stability of IAA to fluorescent and infra-red radiations was studied in the presence and absence of plant tissues. IAA (1 mg. per ml. of water)

TABLE IV
STABILITY OF IAA IN A SERIES OF BUFFERS RANGING FROM pH 1.5 TO 9.0*

Buffer+IAA 10 micrograms	pH	Incubation (hours)		
		1	3	76
		Amount IAA recovered (micrograms)		
Phthalate—HCl	1.5	10	10	< 1
Phthalate—HCl	2.5	10	10	< 3
Phthalate—HCl	3.0	10	10	> 3
Phthalate—NaOH	4.0	10	10	> 8
Phthalate—NaOH	6.0	10	10	> 8
Phosphate—NaOH	9.0	10	10	> 8

* Similar results were obtained when the experiments were done in the presence of plant tissue extracts in the buffers.

in the presence and absence of soybean (*Glycine max* Merr. var. Biloxi) leaf tissue was exposed to fluorescent light of 0.2 g. cal./sq. cm./minute. In a similar series of experiments the same amount of chemical was exposed to infra-red radiation of 1.15 g. cal./sq. cm./minute. Exposure to these radiations was maintained for 180 minutes. At the termination of 5, 10, 30, 60, and 180 minutes, 2.5 microliters of solution were withdrawn from vials containing the solutions of IAA being exposed to the radiations. Paper chromatographs were prepared and the recovery of IAA from solution was calculated by referring to a standard calibration curve. Results of these experiments are given in Table V.

There was little or no destruction of IAA as a result of incubation under fluorescent lights in the absence or presence of leaf tissue for periods up

TABLE V
INFLUENCE OF INFRA-RED AND FLUORESCENT RADIATION ON THE STABILITY OF IAA IN THE PRESENCE AND ABSENCE OF SOYBEAN LEAF TISSUE

Incubation period (minutes)	Soybean leaf tissue	Optical density of spots developed with <i>p</i> -dimethylaminobenzaldehyde on paper chromatographs*	
		Fluorescent 0.2 g. cal./ sq. cm./min.	Infra-red 1.15 g. cal./ sq. cm./min.
5	Present	.28	.26
5	Absent	.28	.28
10	Present	.23	.26
10	Absent	.20	.28
30	Present	.20	.30
30	Absent	.30	.26
60	Present	.24	.18
60	Absent	.27	.26
180	Present	.23	.16
180	Absent	.24	.32

* Theoretical value for total recovery of IAA is .28 to .30.

to three hours. The intensities used in these experiments are much higher than those which are usually encountered under laboratory conditions. Thus, for the relatively short periods of time during which an extraction is done there is little likelihood of inactivation of IAA as a result of radiations from laboratory lights. Infra-red radiation given continuously over one- to three-hour periods partially inactivated IAA only in the presence of plant tissue. In the absence of plant tissue, inactivation of IAA was not observed. That the inactivation was not the result of heating will be apparent from the study involving the effect of temperature on the stability of IAA.

EFFECT OF BOILING IN THE PRESENCE AND ABSENCE OF PLANT TISSUE

It is often desirable to inactivate the enzyme systems which are responsible for catalyzing the synthesis of IAA from tryptophan. Heating plant tissue to achieve destruction of these enzymes was first proposed by Gustafson (4), but later was discarded by other workers who claimed that boiling water released inhibitors of auxin (9). Since the *Avena* coleoptile was the experimental tool employed for auxin determinations, the inhibitors may have had no direct effect upon auxin but instead may have been inhibitory to the growth of *Avena* sections. Therefore, in the present study employing paper chromatography, it was possible to study the effect of boiling directly on the stability of IAA.

The influence of boiling upon the stability of IAA was studied in the manner outlined below:

1. Ten g. of frozen, ground tomato tissue were immersed in 100 ml. of boiling, distilled water for two minutes. Five ml. of the extract were removed and added directly to 40 mg. of IAA. An aliquot (2.5 microliters) of the IAA-boiled tissue mixture was chromatographed on paper (10).

2. IAA was added to the frozen, ground tissue at the rate of 100 mg. to 25 ml. of distilled water and boiled for two minutes. The mixture was filtered and an aliquot (2.5 microliters) was chromatographed.

TABLE VI
STABILITY OF IAA TO BOILING IN THE PRESENCE AND ABSENCE OF PLANT TISSUE
AS DETERMINED BY PAPER CHROMATOGRAPHY

Treatment	Optical density*
Tissue boiled 2 minutes; IAA added to boiled extract	.34
Tissue+IAA boiled 2 minutes	.32
IAA—no tissue—boiled 1 minute	.30
IAA—no tissue—boiled 2 minutes	.29
IAA—no tissue—boiled 10 minutes	.30
IAA—no tissue—boiled 30 minutes	.32

* Theoretical value for total recovery of IAA is .33.

3. Forty mg. IAA were boiled in 25 ml. distilled water for 1, 3, 10, and 30 minutes. At the expiration of these time limits an aliquot from each sample was chromatographed as above.

4. A standard calibration curve for IAA based on optical density of spots developed with *p*-dimethylaminobenzaldehyde served as a reference for the amount of IAA contained in the samples listed in 1, 2, and 3. Results typical of these experiments are recorded in Table VI.

There is no discernible release of inhibitors of IAA from plant tissue which is boiled. These results suggest that boiling plant tissue for short periods may be used to inactivate the enzymes which catalyze the conversion of tryptophan to IAA, providing that IAA content is determined chemically.

DISCUSSION

With the advent of newer methods for determining chemical constituents in living tissues it is necessary to reevaluate the methodology which has been used to date in estimating auxin content of plant tissues. The term auxin itself is symbolic of the nonspecificity which is implicit in biological assays. Sufficient progress has been made with paper chromatographic methods in determining levels of indole compounds in plants that now it should be possible to define the terms free-auxin, bound-auxin, and auxin-complex in precise chemical terms. Before this advance may be achieved it is necessary to use an extraction procedure which will preclude conversion of precursors to the active auxin during extraction, to determine the environmental variables which influence extraction, and to reexamine the current dogmas in this branch of plant physiology. Since auxin has been defined in terms of activity in a biological object (i.e. *Avena* coleoptile) it may include in its definition such diverse chemical structures as naphthaleneacetic acid; 2,4-dichlorophenoxyacetic acid; IAA; 3-indolepyruvic acid; 3-indolebutyric acid; 3-indoleacetonitrile and others. The term auxin has also been used generally to define the naturally-occurring plant growth substance. In some plants the naturally-occurring substance may be IAA, in some it may be 3-indoleacetonitrile, and in others it may possibly be an entirely different compound (6). It is now possible to separate and identify the specific growth substance and to refer to it in precise chemical terms.

The method of quantitative paper chromatography employing density measurements of reactions of indole compounds with *p*-dimethylaminobenzaldehyde will detect 0.1 microgram of IAA. Thus, if large enough amounts of plant tissue are examined for their contents of IAA, it is possible to rely directly upon color development with *p*-dimethylaminobenzaldehyde on paper for quantitative assays. If smaller amounts of tissue are used, it is possible to rely upon qualitative paper chromatography

followed by elution of the substance from the paper and subsequent biological assays. However, in the authors' opinion, the latter procedure introduces additional steps in the method and reduces the quantitative precision of the chromatographic technique. It is more desirable to use greater quantities of plant tissue and maintain quantitative precision. With these considerations in mind, a method of extraction of free IAA from plant tissue, and its subsequent quantitative determination is outlined below. The procedures combine the extraction method (5) described above with quantitative paper chromatography (10).

I. Extraction

1. 300 G. plant tissue harvested and frozen instantly.
2. Frozen tissue ground to fine powder without thawing.
3. Frozen, ground tissue extracted with absolute ethanol for 12 to 24 hours at -10° C.
4. Absolute ethanol evaporated off *in vacuo* leaving aqueous fraction.
5. Aqueous fraction acidified to pH 4.0 with 0.1 N H_3PO_4 .
6. Saturate acidified, aqueous fraction with glucose and extract three times with ethyl ether.
7. Concentrate ether extract to 2.5 ml. or less.

II. Quantitative Paper Chromatography

8. Remove 2.5 microliters of extract and apply to Whatman No. 1 paper as described previously (10).
9. Apply 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 micrograms of IAA (or any other indole compound) to Whatman No. 1 paper.
10. After development of papers, employ density measurements of spots to construct standard calibration curve (10).
11. Calculate amount of indole compound in extract by referring to standard calibration curve.

All operations pertaining to extraction are performed in a cold room (-10° C.). A standard calibration curve should be based on five replicate paper chromatographs that are run simultaneously. The error involved in quantitative paper chromatography of indole compounds in the present tests was ± 3 per cent. The above method has been applied to such diverse plant tissues as soybean (*Glycine max* Merr. var. Biloxi), spinach (*Spinacia oleracea* L. var. Nobel), tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth), and tomato (*Lycopersicon esculentum* Mill. var. Bonny Best). Results with the above techniques showing differences in the types and amounts of indole compounds occurring in photoperiodically-sensitive plants after photoinduction are reported in a succeeding paper of this series (11).

SUMMARY

A comparison was made of two methods for extracting free 3-indoleacetic acid (IAA) from plant tissue. Water extraction of green plant tissues was compared with extractions employing absolute ethanol. The criteria employed to evaluate a suitable technique included: 1) preclusion of conversion of precursors to IAA during extraction, 2) removal of free IAA from plant tissue with one extraction. Removal of free IAA from the tissues was quantitatively determined by the use of paper chromatography. Employing maximum density of spots developed with *p*-dimethylaminobenzaldehyde, it was determined that extraction of quick-frozen, ground tissue with absolute ethanol at -10° C. precludes the conversion of precursors to IAA during extraction and removes free IAA from plant tissue with one extraction. Water extraction of plant tissue followed by ether dissociation of the auxin-complex was found to be unsuitable for quantitative studies.

Several environmental variables which are encountered during the extraction of IAA from plant tissues were evaluated. It was found that IAA is stable up to three hours, either in the presence or absence of plant tissues, at hydrogen ion concentrations ranging from pH 1.5 to 9.0. After 76 hours it is unstable at pH values of 1.5, 2.5, and 3.0. The influence of infra-red and fluorescent radiation upon the stability of IAA was also studied. IAA was found to be stable to fluorescent radiation (0.2 g. cal./sq. cm./min.) up to 180 minutes in the presence or absence of plant tissue. After 60 and 180 minutes of exposure to infra-red radiation (1.15 g. cal./sq. cm./min.), IAA, in the presence of plant tissue, was subject to partial decomposition. The influence of boiling plant tissue as a means of destroying the enzymes which catalyze the conversion of tryptophan and other precursors to IAA was evaluated. It was found that boiling plant tissue in the presence of IAA for short periods of time did not destroy IAA. A method is outlined for the extraction and quantitative determination of free IAA in green plant tissue. The method combines the extraction procedure of Kefford (5) with that of quantitative paper chromatography (10). The need for precise, quantitative terms in studies with naturally-occurring plant growth substances is discussed.

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THE ROLE OF AUXIN IN PLANT FLOWERING. III. FREE INDOLE ACIDS IN SHORT-DAY PLANTS GROWN UNDER PHOTOINDUCTIVE AND NONPHOTO-INDUCTIVE DAYLENGTHS

A. J. VLITOS AND WERNER MEUDT

Paper chromatography as a quantitative analytical method for determining levels of indole compounds in plant tissue has been discussed in previous papers (6, 7). Extraction methods which preclude the conversion of precursors to 3-indoleacetic acid (IAA) during extraction have also been described (4, 7) and have been applied, in combination with quantitative paper chromatography, to the problem of indole chemistry in plants (7).

The present paper deals with the content of indole acids in tissues of short-day plants grown under photoinductive cycles as compared with those in tissues of plants grown under long-day conditions. Quantitative paper chromatography was applied to answer the question: are free IAA levels increased in short-day plants as a result of photoinduction? Such information is critical if plant physiologists are to examine the validity of the hypotheses which have been proposed to account for the influence of auxin in the flowering process (1, 2, 3).

MATERIALS AND METHODS

The short-day plants employed in this study were soybean¹ (*Glycine max* Merr. var. Biloxi and Lincoln), and tobacco (*Nicotiana tabacum* L. var. Maryland Mammoth). Seeds of these crops were planted in a composted sod soil in flats and the emerged seedlings were divided into two groups. One group of plants was grown under greenhouse conditions providing a total of 18 hours' daylength. The prevailing daylength was supplemented by light emanating from 500-watt Mazda tungsten lamps. The second group of plants was grown under short-day conditions (8-hour daylengths) attained by covering the plants with light-proof, aluminum foil shields. Extreme variations in temperature were prevented by the use of aluminum foil. The age of the experimental plant and the number of photoinductive cycles necessary for floral initiation were estimated from previous experiments under these conditions. In every case the plants which were harvested for extraction were photoinduced or were completely vegetative. Photoinduced plants were harvested before visible floral tissue had formed. Vegetative plants were of the same age as photoinduced ones at harvest. The experiments were carried out during the period from September 1953 to April 1954.

¹ The authors are indebted to Drs. W. E. Loomis and David Staniforth of the Iowa State College for supplying seed of Lincoln soybean.

Plants to be extracted were harvested and handled in the following manner: soybean tissue was collected by severing the apical tips of individual plants, including the youngest trifoliate leaf, and freezing the tissue instantaneously by immersion in a beaker held in a mixture of dry ice and methanol. Tobacco tissue was harvested by collecting all of the leaves from individual plants and freezing them immediately. In addition the apical portions of individual plants were severed and frozen in the same manner. Leaf and apical tissues were extracted independently of the other. The frozen tissues were transferred to a cold room (-10°C.) and were

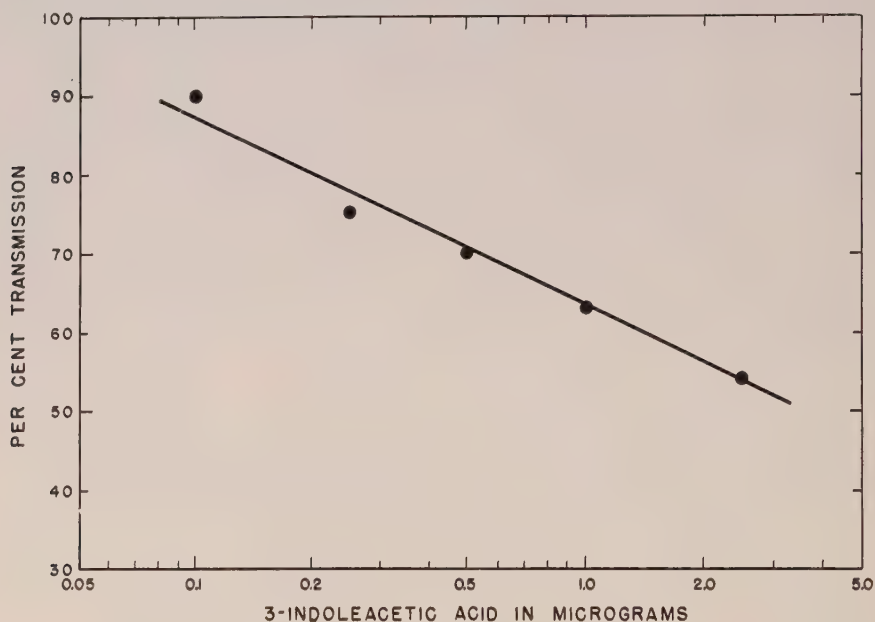


FIGURE 1. A calibration curve for 3-indoleacetic acid based on densitometer readings of spots developed with *p*-dimethylaminobenzaldehyde on paper chromatograph.

passed through a meat grinder. The fine powder which was obtained did not thaw during or after the grinding operation. After 300 g. of frozen, ground tissue were collected from the individual short- and long-day sets of plants, 500 ml. of chilled absolute ethanol were added to each 300 g. of tissue. Approximately 12 hours later the absolute ethanol extract was centrifuged free of plant tissue, and three successive rinsings of the residual tissue were combined with the original ethanol extract. The ethanol was evaporated off under reduced pressure, leaving an aqueous residue. The aqueous fraction was centrifuged at 10,000 r.p.m. for 15 minutes to free the extracts of residual chloroplasts. The above operations were performed in a cold room (-10°C.). The aqueous fraction was adjusted to pH 4.0 by the addition of 0.1 *N* phosphoric acid, and then saturated with glucose.

Three successive extractions of the glucose-saturated, acidified, aqueous fraction with ethyl ether (peroxide-free) yielded the final extract. The ether extracts were reduced in volume *in vacuo* to between 1.0 and 2.5 ml. Quantitative paper chromatography of the ether extracts was accomplished as described previously (6). Known amounts of IAA (0.1 to 10.0 micrograms) were chromatographed simultaneously with the extracts. Optical density of spots developed with *p*-dimethylaminobenzaldehyde plotted against the concentration of IAA gave a straight-line relationship (Fig. 1). The amounts of IAA present in the extracts were determined by extrapolation.

EXPERIMENTAL RESULTS

The kinds and amounts of free indole compounds recovered from tissues of Biloxi soybean, Lincoln soybean, and Maryland Mammoth tobacco in typical experiments are listed in Table I.

TABLE I

AMOUNTS OF 3-INDOLEACETIC ACID (IAA), 3-INDOLEPYRUVIC ACID (IPA), AND TRYPTOPHAN (TRYP.) IN SHORT-DAY PLANTS GROWN UNDER 8- AND 18-HOUR PHOTOPERIODS AS DETERMINED BY QUANTITATIVE PAPER CHROMATOGRAPHY

Tissue	Photoperiod (hrs.)	Micrograms/300 g. of tissue		Tryp.** (Rf 0.46)
		IAA (Rf 0.51)	IPA (Rf 0.21)*	
Biloxi soybean	8	125.0	+++	Not determined
Biloxi soybean	18	<0.1	+	Not determined
Lincoln soybean	8	0.1 to 0.25	+++	Not determined
Lincoln soybean	18	<0.1	o	Not determined
Maryland Mammoth tobacco leaves	8	<0.1	++	++
leaves	18	<0.1	o	++
apices	8	<0.1	o	o
apices	18	<0.1	o	++

* It was not possible to express the amounts of 3-indolepyruvic acid quantitatively since synthetic samples of the compound were too unstable to chromatograph quantitatively.

** Tryptophan was contained in the aqueous fractions of tobacco extracts. Soybean extracts were not assayed for tryptophan content.

A compound possessing an Rf value identical to 3-indolepyruvic acid (IPA) was detected in the three short-day plants which were examined. In all cases, strongly colored spots on paper chromatographs were obtained for this compound in extracts from tissues of plants grown under 8-hour daylengths. A synthetic sample of IPA² was found to be highly unstable, being converted into IAA and tryptophan. The Rf value of 0.21 for the

² 3-Indolepyruvic acid was supplied through the courtesy of Mr. Joseph Lambrecht and Dr. Thomas Fitzpatrick, Carbide and Carbon Chemicals Co., South Charleston, West Virginia.

synthetic sample corresponded to the Rf value obtained from tissue extracts. Free IAA was present in highest concentration in extracts of tissues of Biloxi soybean plants grown under short-day conditions. Lincoln soybean plants grown under short-day conditions also contained greater amounts of free IAA than comparable tissue from plants exposed to 18-hour photoperiods. Maryland Mammoth tobacco leaves grown under 8-hour photoperiods contained more IPA than IAA. Tobacco apices were devoid of IPA and contained less than 0.1 microgram of IAA per 300 g. of tissue. The tryptophan present in tobacco tissue was obtained in the aqueous fraction of the extract.

DISCUSSION

Stowe and Thimann (5) have described the occurrence of IPA in extracts of maize. The compound also has been detected recently in tomato tissues treated with tryptophan (7). Its abundance in the photoperiodically-sensitive plants described above is of considerable interest considering the key role which IPA might play as a precursor of IAA. One of the reasons why 3-indolepyruvic acid may have escaped detection in green plants in the past is probably the fact that bioassays have been used so widely in quantitative auxin determinations. It is quite possible that the growth activity of plant extracts, assayed by means of the *Avena* coleoptile, may represent the combined activity of IAA and IPA. In other words, the term auxin is ambiguous because it may refer to unknown compounds in plants and to many compounds which are known synthetic plant growth regulators.

Tissue extracts, containing the compound which is tentatively described as IPA, continued to yield heavily-colored spots at Rf 0.21 after several weeks of storage at 5° C. Synthetic samples of IPA, however, were highly unstable, confirming the report of Stowe and Thimann (5). A stable salt or ester of IPA would be of value in assaying the role of this compound in plant growth. The reasons for the greater stability exhibited by IPA in tissue extracts as compared to synthetic samples have not been ascertained.

The occurrence of greater amounts of free IAA in the tissues of Biloxi soybean plants grown under 8-hour photoperiods is of interest, especially since Lincoln soybeans under comparable growth conditions contain much more IPA than IAA. Some workers have postulated that auxin levels are decreased during photoinduction and have suggested that flowering in short-day plants is favored by low auxin levels (1), or by a balance between auxin and florigen (2). The results of the present study indicate that free IAA levels are increased under light conditions favoring the flowering Biloxi soybeans. In this connection it should be emphasized that only the free indole acids were isolated by the present methods. The amounts of

IAA bound to proteins or of IAA which might be formed from available precursors were not determined in the present study. These forms of IAA are also subject to study by means of quantitative paper chromatography. Further studies involving these other forms of potential IAA are being pursued. It is also conceivable that differences in levels of free indole acids between plants grown under different daylengths may be a phenomenon dependent on daylength but not directly involved in photoinduction.

SUMMARY

Free indole acids extracted from tissues of short-day plants grown under photoinductive and nonphotoinductive daylengths were examined by means of quantitative paper chromatography. 3-Indolepyruvic acid was found in leaves of Biloxi and Lincoln soybeans and in leaves of Maryland Mammoth tobacco. In every case greater quantities of 3-indolepyruvic acid were detected when plants were grown under photoinductive daylengths. Free IAA was found to be present in greater amounts in tissues of Biloxi than in Lincoln soybeans grown under short-day conditions. The view is expressed that free IAA may not have a direct effect on photoinduction or floral initiation in short-day plants, but its synthesis may be influenced by length of day.

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EFFECT OF ANTIBIOTICS ON PLANT GROWTH

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The favorable effect of some antibiotics on animal growth has led to an investigation of the effects of these substances on the growth of higher plants. Various reports have indicated that antibiotics have an adverse effect on plant growth. It was noted in 1946 (22) that penicillin delayed the germination of French lettuce seeds in contact with the solution. Euler also reported early (7) that streptomycin at a concentration of 1 mg./ml. inhibited growth and chlorophyll formation in seven different kinds of plants, including bean, lettuce, and radishes, as well as some grasses. More recently Nétien *et al.* (16) have demonstrated the same type of response of lentils, peas, and rape to Aureomycin (chlortetracycline) and Terramycin (oxytetracycline). Other work has also pointed to the inhibitory effects of antibiotics. For example, it has been shown that reduction of wheat, clover, and mustard root growth occurred in Petri dishes on tap-water agar containing various antibiotics at concentrations of 25, 5, and 1 p.p.m. (27). Also, the main rootlets of corn were markedly inhibited when they were grown on filter paper moistened with solutions containing 50 to 1500 p.p.m. of Terramycin (24). Cercos (5) found that the antibiotics, fungocin and bacilomycin, inhibited mainly the rootlets and root hairs of wheat.

Nickell (18), on the other hand, obtained evidence of the stimulation by antibiotics of the growth of virus tumor tissue *in vitro*, and the germination of seeds and early growth of seedlings of *Agave toumeyana*, narrow-leaved sorrel, radish, cucumber, and sweet corn. He pointed out that such effects were to be found only at very low concentrations (1 to 10 p.p.m.) of the antibiotics, and that the use of higher concentrations by some other workers may have caused the inhibitions reported by them. In corroboration of Nickell's work, it was reported (2) that growth increases of as much as 75 per cent were secured at the University of California with bean seedlings grown in garden soil containing a few parts per million of Terramycin; and that tomatoes grown in sand sub-irrigated with mineral nutrients showed growth increases of about 25 and 55 per cent as a result of adding penicillin and Aureomycin. However, U.S.D.A. workers at Beltsville, Md. (20) failed to confirm beneficial effects of Terramycin in preliminary tests on hybrid corn, radishes and beans. Hervey (11) soaked seeds of oats, Buffalo grass, and Madrid sweet clover and then watered the plants at five-day intervals after emergence with solutions of bacitracin, Terramycin, thiolutin, and actidione. All produced increased germination,

¹ The authors wish to express their appreciation to Chas. Pfizer & Co., Inc., Brooklyn, N. Y. for supplying antibiotics and financial support, in part, for this investigation.

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sprout growth and total weight. He attributed the stimulation to possible increased microbial growth. Harris (10) watered young corn plants with 0.5 to 10 p.p.m. of antibiotics and found that potassium penicillin at 5 p.p.m. increased the dry weight by 40 per cent, but that procaine penicillin decreased the dry weight by 20 per cent. He thought that possibly potassium and procaine were the effective agents. The beneficial effect of all other antibiotics tried was slight.

More recently, Nickell and Finlay (19) have obtained definite stimulation of the growth of the duckweed, *Lemna minor*, by the addition of certain antibiotics under aseptic conditions.

The present paper reports the effect of nine different antibiotics on the growth of wheat roots in solution. The effect of some of them, especially Terramycin, on seed germination and growth of certain seedlings in the greenhouse and field is also described.

GROWTH OF WHEAT ROOTS IN SOLUTION

MATERIALS AND METHODS

The following antibiotic preparations furnished by Chas. Pfizer & Co., Inc. were used:

Bacitracin 51 units/mg.	Streptomycin sulfate 765 γ /mg.
Catenulin hydrochloride	Streptomycin tri hydrochloride 790 units/mg.
Magnamycin (carbomycin) 1080 units/mg.	Terramycin (oxytetracycline) hydrochloride 890 γ /mg.
Potassium penicillin G 1,000,000 units	Terramycin, crude 502 γ /mg.
Polymyxin B sulfate 7100 units/mg.	Thiolutin 1000 γ /mg.
Sodium rimocidin	

Wheat roots were grown in solutions of these antibiotics made up in different concentrations in three different solvents, distilled water, tap water, and a three-salt nutrient solution. The latter contained 0.02 *M* potassium dihydrogen phosphate, 0.02 *M* calcium nitrate, and 0.02 *M* magnesium sulfate, a solution which has been shown to be favorable for the growth of wheat roots (3). It was found very early in the tests that tap water permitted as much growth of wheat roots as the nutrient solution just described. Consequently, the nutrient solution was used for comparison with distilled water in the first tests only, after which tap water was used instead of the nutrient. The concentrations of the solutions were calculated using the potencies listed above, and considering γ and units as the same. Catenulin hydrochloride and sodium rimocidin potencies were not known, so these were treated as pure substances in making the solutions, i.e. 32 mg. per liter for 32 p.p.m. concentration. With the available information on potencies of the materials used, it is obviously not possible

to make direct comparisons, but certain relative effects may be determined. Miles (15) points out the need for standardization of units for expressing potencies of antibiotics which vary with each lot produced.

For the most part, the antibiotic materials were easily dissolved in water. It was necessary to heat thiolutin and Magnamycin to get them into solution, and sodium rimocidin and crude Terramycin did not dissolve completely at a concentration of 32 p.p.m. The growth of the primary root of the young wheat seedlings in the solutions was taken as a measure of the inhibiting or promoting effect of the material tested.

The technique for growing wheat roots in solution has been described previously (3). All solutions to be tested were transferred to beakers which had previously been prepared in the following manner. The mouths of 300-ml. Berzelius beakers were covered with paraffined cheesecloth in which 25 small holes were punched. A paraffined thread was used to tie the cheesecloth securely over the beaker. This having been done, the cheesecloth was trimmed and each 300-ml. beaker was placed inside a 600-ml. one of the same form.

The solutions were then poured in until the small beaker and the space around it were entirely filled, the level being even with the top of the small beaker, so that no air bubble was left under the cheesecloth. Care was taken that the level of the solution did not extend above the cheesecloth.

In the bottom of glass-covered culture dishes (about 24 cm. in diameter) were placed sheets of filter paper, which were then moistened with distilled water. Into the dishes were placed grains of wheat (*Triticum* sp.)², which had previously been soaked in distilled water for three to four hours and rinsed several times to remove foreign matter which might be present. The grains were taken individually with forceps and placed furrow side down. The dishes were placed at 25° C. until the primary root had attained a length of about 6 mm. (usually 24 to 28 hours). The germinated grains were then placed on the paraffined cheesecloth, so that the primary roots dipped into the solutions. Before each grain was placed in contact with the first solution, the length of the primary root was recorded. Then a grain with a primary root of the same length was placed in each of the other cultures of the series. This process was repeated until there were 25 actual measurements of primary root lengths. An average of these lengths was taken as a measurement of the initial root length. Duplicate cultures of 25 seedlings each were used for each treatment for most of the tests.

The cultures were then placed in a dark chamber at 20° C. During the first 48 hours, each culture was covered with a watch glass. The duration of the growth period was about 72 hours, after which time the cultures

² The wheat seeds used throughout the tests were Marquis, 1952 crop, from the North Dakota Agricultural Experiment Station, Fargo, N. D.

were removed from the incubator and the length of the primary root of each seedling was recorded. Each of the growth values given in the tables was obtained by deducting the mean initial root length from the mean final root length. This value for elongation was expressed in mm. or as a percentage of the average elongation for the control, i.e. distilled water, tap water or nutrient solution to correspond to the solvent used for the antibiotic in the test solution.

RESULTS AND DISCUSSION

Effect of water source. A comparison of the growth response of wheat roots in the presence of nine different antibiotics dissolved in distilled water, tap water, or nutrient solution is shown in Table I. Two forms of streptomycin, the sulfate and the tri hydrochloride, and two forms of

TABLE I
GROWTH OF WHEAT ROOTS IN SOLUTIONS OF ANTIBIOTICS

Antibiotic	Medium*	Wheat root growth (% of control) in γ or units/ml. of antibiotic					
		32.0	10.0	3.2	1.0	0.32	0.1
Bacitracin	D	0	12	32	94	149	133
	T	58	75	80	87	90	87
Polymyxin	D	5	26	98	127	134	101
	T	8	57	102	100	99	100
Potassium penicillin G	D	1449	1491	1512	1192	504	1353
	T	101	114	105	108	109	107
Streptomycin tri hydrochloride	D	5	5	64	417	318	335
	N	101	99	98	95	94	93
Terramycin hydrochloride	D	0	117	124	91	89	130
	N	57	59	65	89	93	97
Thiolutin	D	0	0	0	23	134	77
	N	0	0	0	4	60	95
Catenulin hydrochloride**	D	0	0	7	10	16	38
	T	0	0	4	17	55	136
Magnamycin	D	102	96	66	38	37	38
	T	114	100	110	106	100	101
Sodium rimocidin**	D	32	87	90	108	94	65
	T	27	75	100	97	97	95
Streptomycin sulfate	D	0	0	109	106	101	126
	T	51	58	70	101	111	111
Terramycin, crude	D	0	36	69	97	91	117
	T	3	8	19	93	105	109

* D = distilled water; N = nutrient solution; T = tap water.

** Potency not known. 32 Mg./liter used as 32 p.p.m.

Terramycin, the hydrochloride and the crude form, were used. Crude Terramycin is a commercial preparation of about 50 per cent activity.

It will be noted that the substances tested could be divided into three fairly definite groups as measured by their effects on the growth of wheat roots in solution. Bacitracin, potassium penicillin G, polymyxin, strepto-

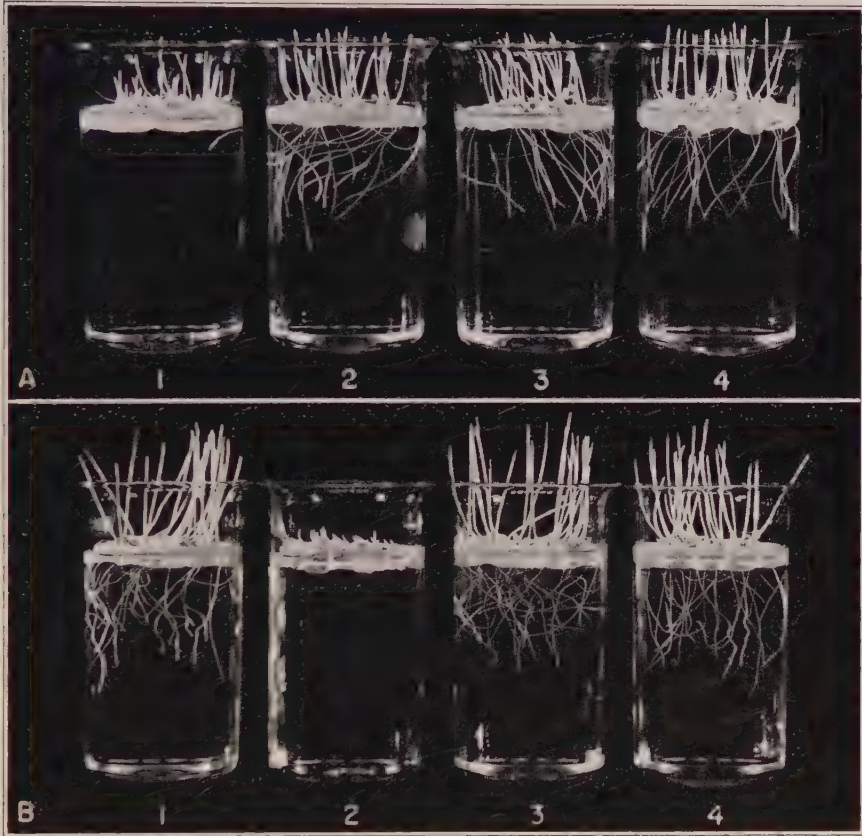


FIGURE 1. Growth of wheat roots in solution. A. Effect of penicillin added to distilled water A at (1) 0, (2) 100.0, (3) 32.0, and (4) 3.2 p.p.m. B. Effect of (1) distilled water B, (2) distilled water A, (3) double distilled A, and (4) demineralized A.

mycin tri hydrochloride, Terramycin hydrochloride, and thiolutin enhanced the growth of wheat roots at some concentrations when distilled water was used as a medium. However, the percentage increase in growth varied greatly depending upon the antibiotic added to the solution. For example, potassium penicillin G permitted more than a tenfold increase in root length when added to distilled water in concentrations ranging from

32.0 to 0.1 p.p.m., with the exception of 0.32 p.p.m., which, for some reason, permitted only half that growth. Potassium penicillin G was far less toxic than the other antibiotics, for it continued to benefit wheat root growth in solutions as strong as 320 p.p.m., the strongest tried. Some of these effects are shown in Figure 1 A.

Next to penicillin, streptomycin tri hydrochloride brought about the greatest increase in root length in distilled water (up to 417 per cent), but at low concentrations only. Streptomycin sulfate did not have this effect. It will be seen also that bacitracin, polymyxin, Terramycin hydrochloride and thiolutin all increased wheat root length in distilled water. Later tests of Terramycin hydrochloride showed from 217 to 337 per cent increase in the length of wheat roots growing in 10 p.p.m. in distilled water.

The addition of the antibiotics included in this first group to either nutrient solution or tap water failed to bring about an increased growth of the wheat roots. Even potassium penicillin G, which produced such a beneficial effect in distilled water, permitted only 101 to 114 per cent of the growth in tap water.

In contrast to the first group, the second group of antibiotics represented by catenulin and Magnamycin failed to enhance the growth of wheat roots in distilled water, but showed decreased growth at some concentrations as compared to the same concentrations in tap water (Table I). It should be pointed out that growth in distilled water alone was very poor, while that in tap water alone was good. It will be noted that the toxicity of Magnamycin to wheat roots was increased with decreasing concentration of the antibiotic in distilled water. The reason for this response is not known.

In the third group of antibiotics, sodium rimocidin, streptomycin sulfate, and crude Terramycin, it will be seen (Table I) that the effect on growth of wheat roots is about the same in distilled or tap water.

All of the results in the distilled water series shown in Table I were obtained using water (A) from the same source, a Barnstead still. Growth of wheat roots in this water varied from time to time, but was always very poor. This is shown in Figure 1 A, where it will be seen that some coleoptile growth occurred in spite of the root inhibition. In later series, water from this source was demineralized or redistilled in glass before using. Also, distilled water (B) from the same type of still in another laboratory was used. Growth of wheat roots in the four types of distilled water is shown in Figure 1 B. Distilled water B (Fig. 1 B, 1) permitted much greater elongation of the roots than distilled water A (Fig. 1 B, 2). Demineralization or redistilling in glass (Fig. 1 B, 3 and 4) removed the toxic effect of distilled water A. Heavy metal analyses of distilled waters A and B, demineralized A, and the tap water used were made by Chas. Pfizer & Co., Inc. with the following results:

Water	Heavy metals γ /100 ml.			
	Zinc	Lead	Copper	Iron
Distilled A	2	2	5	4
Distilled A (demineralized)	1	1	4	0
Distilled B	2	3	0	0
Tap	22	4	4	12

The residue of the tap water was largely composed of silica, magnesium, and calcium.

Further tests in which Terramycin at 32.0, 10.0, and 3.2 p.p.m. was used in the different types of water showed that the Terramycin effect was independent of the type of distilled water used (A, B, or demineralized A) when the absolute length attained by the roots was considered. For example, 32.0 p.p.m. Terramycin permitted only 1 mm. and 10.0 p.p.m. only 26 mm. of growth in all three distilled waters. That some toxic effect is exerted by distilled water A is evident, and it is also apparent that this effect does not influence the response of wheat roots to Terramycin dissolved in water. Viewed from another angle, Terramycin at 10.0 and 3.2 p.p.m. overcomes the harmful effect of distilled water A, permitting a five- to sevenfold increase in elongation of wheat roots. However, Terramycin of the same concentrations in distilled water B or demineralized A inhibited elongation about 50 per cent.

Effect of heavy metals. It is doubtful whether the presence of either iron or copper can account for the toxicity of distilled water A since additions of greater amounts of either or both of these metals to distilled water B failed to injure wheat roots. However, it will be noted that the principal metal removed by demineralization of distilled water A was iron, and since Terramycin is known to act as a chelating agent (21), preliminary experiments were performed to see whether this property might account for some of the effects on wheat root growth. The stimulating effect of the tetra sodium salt of the chelating agent, ethylenediaminetetracetic acid (EDTA) on wheat root growth (Fig. 2 A) supported this possibility. EDTA overcame the inhibiting action of distilled water A, but failed to stimulate growth in tap water, an effect similar to that of some antibiotics. In concurrent tests with those made in this laboratory, stimulation of the growth of *Lemna minor* by EDTA was secured by Nickell and Finlay (19).

Iron and copper are heavy metals which often form complexes with certain chelating agents. Since the toxic distilled water A contained more iron than any other distilled water used, an experiment was performed using combinations of Terramycin and ferric chloride in contact with wheat roots in solution. Some of the effects are shown in Table II. Ferric chloride (FeCl_3) in concentrations of 10^{-4} to 10^{-8} M reduced the growth



FIGURE 2. Growth of wheat roots in solutions made with distilled water A. A. Effect of the sodium salt of ethylenediaminetetracetic acid at (1) 0, (2) 2.13, (3) 0.53, and (4) 0.108 p.p.m. B. Effect of 10^{-5} M FeCl_3 in combination with EDTA at (1) 0, (2) 2.13, (3) 0.53, and (4) 0.108 p.p.m. C. Effect of a combination of bacitracin and penicillin: (1) distilled water control, (2) 0.32 p.p.m. bacitracin, (3) 3.2 p.p.m. penicillin, and (4) solution containing 2.4 γ /ml. penicillin and 0.08 unit/ml. bacitracin.

TABLE II
GROWTH OF WHEAT ROOTS IN SOLUTION. GROWTH EXPRESSED AS PER CENT
OF THE DISTILLED WATER CONTROL

Terramycin hydrochloride, p.p.m.	FeCl ₃ (M)					
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	0
32.0	0	0	9	0	0	0
10.0	0	21	246	260	274	278
1.0	0	88	100	82	110	87
0.0	0	58	53	64	73	100

of wheat roots in toxic distilled water A. Terramycin at 10 p.p.m., on the other hand, increased the root length over the control by more than two-fold when used alone or in combination with 10⁻⁶, 10⁻⁷, or 10⁻⁸ M FeCl₃. However, 10.0 p.p.m. did not totally prevent the injury to wheat roots of 10⁻⁵ M FeCl₃. The last-named combination of Terramycin and FeCl₃ permitted only 21 per cent of the elongation of that in distilled water, while in the Terramycin or FeCl₃ alone the percentages of growth were 278 and 58 per cent respectively. This suggests the possible formation of a Terramycin-iron complex which is more toxic than either of the component parts.

At 32.0 p.p.m., Terramycin hydrochloride is toxic alone and with all of the iron concentrations tried. That the iron toxicity is removed by 1.0 p.p.m. Terramycin hydrochloride, however, is shown by increased growth of wheat roots in the iron solutions in the presence of the antibiotic (Table II). The combined effects of Terramycin hydrochloride and ferric chloride are not due to acidity, as shown by pH measurements of the solutions at the beginning and end of the experiment. Similar tests using copper or combinations of copper and iron with Terramycin hydrochloride yielded essentially the same results.

The favorable effects of EDTA on root growth are also reduced by the addition of FeCl₃ at 10⁻⁵ M (Fig. 2 B). Furthermore, as the concentration of EDTA is reduced from 2.13 to 0.108 p.p.m., 10⁻⁵ M FeCl₃ has a greater depressing effect on root growth. It should be noted, however, that whereas EDTA shows a simple chelating effect, i.e. it combines with iron ions to form complexes in which the iron is nonionic, Terramycin hydrochloride shows, in addition to this, an apparent formation of a toxic complex. Albert (1) has noted that Terramycin has a high affinity for metallic ions and that the antibacterial quality of 8-hydroxyquinoline depends upon its ability to combine with metals. Also, following studies of its chelating properties with iron and copper, Waggoner and Dimond (25) concluded that lycomarasmin present in culture filtrates of *Fusarium* must act as an iron complex to form the vivotoxin in *Fusarium* wilt of tomatoes.

Effect of nontoxic cations. Newton (17) found that the addition of nontoxic cations, such as magnesium and calcium, to solutions of polymyxin rendered the antibiotic inactive against *Pseudomonas aeruginosa* due either to competitive absorption or to the formation of a polymyxin-metal complex. In this case, the complex should remove the harmful effect rather than increase it as in the one instance with Terramycin noted above. More recently, Rosen (23) found that the manganous ion, either as chloride or sulfate, did not reduce streptomycin inhibition of the growth of *Escherichia coli* "B" in liquid culture. On the other hand, the same ion prevented the streptomycin inhibition of coleoptile elongation. Rosen found calcium ions only half as effective as manganese, and the other tested cations ineffective.

TABLE III
INFLUENCE OF CALCIUM AND MAGNESIUM ON THE EFFECT OF TERRAMYCIN
HYDROCHLORIDE ON WHEAT ROOTS IN SOLUTION

Terramycin hydrochloride, p.p.m.	Average growth in mm. of wheat roots per culture				
	Control	Ca(NO ₃) ₂ (M)		MgSO ₄ (M)	
		.000025	.00025	.000025	.00025
0*	53	59	44	12	4
3.2	35	41	35	24	4
10.0	27	37	34	18	5
32.0	1	34	31	14	7

* Distilled water B.

To test the possible role of nontoxic cations in the mechanism of Terramycin action, calcium nitrate and magnesium sulfate were added to solutions of the antibiotic and the effect on the growth of wheat roots was measured. The concentrations used and the results obtained are to be found in Table III. It will be noted that distilled water B, which permitted an elongation of 53 mm. in wheat roots during the incubation period, was used throughout this test. This means that the three concentrations of Terramycin used, i.e. 3.2, 10.0, and 32.0 p.p.m., caused a reduction in wheat root growth over that of the control. The figures given in the table represent averages of the actual measurements of 25 roots from each culture.

The addition of the calcium ions reduced the injury to wheat roots by 10.0 and 32.0 p.p.m. Terramycin, but permitted no increased growth over that in distilled water. The lack of effectiveness of magnesium in removing toxicity may have been due to the failure to secure the right concentration of this ion.

Effect of combinations of antibiotics. Limited studies have been made of possible additive, synergistic or antagonistic effects on wheat root growth

of combinations of antibiotics. Concentrations of each previously determined as most likely to enhance growth were used. Combinations of bacitracin, penicillin, polymyxin, streptomycin, and Terramycin hydrochloride, each with the other, were tested.

A solution containing 0.08 unit/ml. of bacitracin and 2.4 γ /ml. of penicillin increased root length over that secured with either alone in distilled water A (Fig. 2 C). This seems to be a simple additive effect. All other antibiotic combinations were without effect.

Antibiotic synergism, antagonism, and indifference have all been reported in combination effects on bacterial resistance (6, 9). Furthermore, a study of the antibiotic effect on auxin action on *Avena* coleoptiles and cucumber seedling development has revealed both synergism and an inhibition of auxin action depending upon the antibiotic used (12). In tests made in this laboratory on wheat roots, the toxic effect of 1.0 p.p.m. 3-indoleacetic acid could be partially overcome by penicillin, Terramycin, and thiolutin, the only antibiotics tested. No complete reversal of auxin action or synergism was noted under these conditions.

Effect of age of antibiotic solution. Terramycin solutions left standing in the laboratory become yellow in color, and are known to lose their antibiotic activity. An experiment, where a comparison was made between fresh and one-week-old 10.0 p.p.m. solutions, showed greater increased growth of wheat roots in the latter as compared with the controls in distilled water A. The difference is probably due to some factor present or absent at the very beginning of the test, i.e. when the wheat roots are first placed in the solutions, since they are allowed to grow in the solutions for three days, during which time some degradation has occurred, before they are harvested.

The possible favorable effect of some degradation product of Terramycin is indicated. Nickell and Finlay (19), following their finding of increased growth of *Lemna* in old solutions, tested a number of degradation products of Terramycin and found stimulation, inhibition, or no effect. Root growth-stimulating factors of penicillin are not removed by inactivation of the antibiotic according to Weihe (26) who states that Bein, Signer, and Schopfer attributed the stimulation to impurities such as β -indoleacetic acid. Other workers have thought that certain inhibiting effects are due to the same impurity (4, 10, 14). Clearly, more work is needed on this phase of the problem.

GERMINATION OF SEEDS AND GROWTH OF SEEDLINGS

Although the major portion of the present work to determine the effect of antibiotics on plant growth was done with wheat roots in solution as described above, limited tests of their effect on the germination of seeds and growth of seedlings were conducted. The results are discussed briefly in this section.

Effect on germination. Cucumber (*Cucumis sativus* L.) and wheat (*Triticum* sp.) seeds were placed to germinate on filter paper moistened with distilled water solutions of different concentrations of Terramycin hydrochloride, polymyxin, Magnamycin, and streptomycin tri hydrochloride. There was some hastening of germination in the very early stages upon addition of any one of these substances in concentrations of 10 p.p.m. or lower. This was evident after one day for cucumber and after two days for wheat. After longer periods, the initial advantage disappeared so that no differences were apparent. When the filter paper was moistened with nutrient solution germination was retarded, and the addition of antibiotics seemed to counteract this inhibition to some extent.

The effect of Terramycin hydrochloride in concentrations of 0.1, 0.32, 1.0, 3.2, 10.0, and 32.0 p.p.m. on the absorption of water by cucumber seeds was tested during periods of one-half, one, two, four, eight, and sixteen hours. Weights of air dry seeds in duplicate lots of 50 seeds each were recorded before soaking. After removal from the solutions, excess moisture was removed from the seed surfaces before they were placed in the weighing bottles. There were no differences in the amount of water absorbed during any time period by seeds soaked in Terramycin solutions and those soaked in distilled water. Thus it appears that antibiotics do not affect the permeability of seeds to water.

Seeds of corn (*Zea mays* L.), cucumber, and tomato (*Lycopersicon esculentum* Mill.) were presoaked in concentrations of 0, 50, and 500 p.p.m. of Terramycin for 4 and 16 hours at 10°, 20°, and 30° C., after which they were germinated on filter paper or in soil moistened with water. There was no significant effect on germination but the corn soaked 16 hours in Terramycin at 500 p.p.m. produced many chlorotic plants which failed to survive in soil in the greenhouse.

Effect on seedling growth in the greenhouse. The effect of Terramycin on the growth of plants in the greenhouse was tested by adding the antibiotic to the soil. Plantings of seeds of alfalfa (*Medicago sativa* L.), carrot (*Daucus carota* L. var. *sativa* DC.), corn, cucumber, soybean (*Glycine max* Merr.), spinach (*Spinacia oleracea* L.), tomato, and wheat were made in three types of soil, good loam, poor sod soil, and river sand. Terramycin at concentrations of 32.0, 10.0, 3.2, 1.0, 0.32, and 0.1 p.p.m. was used to water the seeds as needed until germination was complete. Thereafter, solutions were added to the pots once each week, supplemented with water as needed. Fresh solutions were made for each watering. Watering with tap water alone served as a control. The seeds were planted in glazed crocks in replicates of five each arranged in balanced incomplete blocks. Twenty-five seeds each of corn, cucumber, and wheat and 50 each of alfalfa, carrot, soybean, spinach, and tomato were planted. Seedling counts were made each day as long as there was an increase in number. Records of

death by disease, usually damping-off, were also taken. As soon as germination was complete all cucumber and corn plants, except the one nearest the center of the crows, were removed. Similarly, tomato plants were thinned to two per crows, and carrots to five per crows. No thinning was done in the crows of alfalfa, soybean, spinach, or wheat. Wet and dry weights of the tops of all plants were determined after growing for two or three months in the greenhouse. In addition, fruit production was measured for cucumber and tomato. Hand pollinations were made on all cucumber and tomato flowers as they opened for a period of 21 and 28 days respectively. The cucumber fruits were harvested as they reached marketable size, and the length and weight of the individual fruit recorded. Tomato fruits were harvested and weighed as they ripened.

There was no increase in survival or growth of the seedlings as a result of applying Terramycin to the soil. Similarly, cucumber fruits showed no significant differences in size due to the antibiotic.

TABLE IV
YIELD OF TOMATO FRUITS IN GOOD SOIL IN THE GREENHOUSE

Terramycin hydrochloride, p.p.m.	Weights (g.) of ripe fruits per row for harvest periods											
	April 13 to April 30 incl.						April 13 to May 13 incl.					
	1	2	3	4	5	Totals	1	2	3	4	5	Totals
32.0	151	74	307	429	234	1195	888	519	825	1098	689	4019
10.0	118	287	373	283	586	1647	728	843	925	740	983	4219
3.2	60	142	170	294	399	1065	447	592	794	884	903	3620
1.0	96	160	250	561	424	1491	331	569	1010	821	1130	3861
0.32	367	380	305	183	383	1618	775	825	672	905	956	4133
0.1	193	73	198	280	185	929	764	713	556	698	653	3384
0.0*	110	87	300	121	257	875	606	508	667	418	733	2932
Totals	1095	1203	1903	2151	2468	8820	4539	4569	5449	5564	6047	26168

* Tap water control.

The tomato fruit yield in each of the five blocks is shown in Table IV. The first fruits ripened on April 13, and the left half of the table shows the fruit production up to April 30, while the right half of the table shows the total harvest up to the time the plants were discarded on May 13. Watering the tomato plants with certain concentrations of Terramycin hydrochloride seems to result in earlier maturity of fruits, when the totals alone are considered; 875 grams from the tap water controls as compared with 1647 grams from plants watered with 10 p.p.m. of the antibiotic. However, a study of the wide variations in the yields of the individual plants explains the lack of significance of these differences, a fact borne out by statistical analysis. The "near-significance" of the figures may be suggestive for future study especially since the same relationship holds for the total weights of fruit for the entire harvest period of April 13 to May 13.

It should be pointed out that it is difficult to evaluate effects in the soil because of the possible inactivation of the antibiotics by acidity, biological activity, ionic adsorption, or chemicals (8, 13).

Effect on seedling growth in the field. The effect of spraying solutions of Terramycin hydrochloride on the foliage was determined for field plantings of cabbage (*Brassica oleracea* L. var. *capitata* L.), tomato, carrot, and snapdragon (*Antirrhinum majus* L.). These plants were selected because the marketable portions are leaves, fruits, roots, and flowers respectively. For each kind of plant there were five Latin squares. For cabbage and snapdragons, two plants were set at each of the 25 different locations of each square, making a total of 50 plants for each of the five treatments; for tomato, only one plant was set at each location, making a total of 25 plants for each treatment; and for carrot, the plants were thinned after germination to leave six at each location, or a total of 150 plants for each treatment. Cabbage, snapdragon, and tomato seeds were started in the greenhouse and seedlings were transplanted to the field. Carrot seeds were sown directly in the field and were thinned after the seedlings had appeared.

The plants were given three sprays at intervals of approximately two weeks with concentrations of 10, 50, 100, and 500 p.p.m. of Terramycin hydrochloride. The controls were sprayed with water. In all cases, the seedlings were allowed to become established before the first spray was given. Cabbage, snapdragon and tomato plants received their first spray after one month, two weeks, and three weeks in the field. Carrots were sprayed about one month after the seeds were planted. Tap water was used for the first spray solutions and distilled water for the later ones. A measured amount of solution of sufficient quantity to cover the entire plant to run-off was sprayed through a DeVilbiss spray gun connected to an electric air compressor which produced about ten pounds' pressure.

All of the cabbage was harvested at the same time, i.e. when most of the heads had reached marketable size; snapdragon flowers were cut and counted as they appeared; carrot roots were weighed when most of them had reached marketable size; and tomato fruits were counted and weighed as they ripened. There were no significant differences in the yields due to treatment, though there was a significant difference in the performance in the different blocks. There was a slight chlorotic effect of the 500 p.p.m. concentration.

In conclusion, then, it can be stated that Terramycin as a spray had no favorable effects on the growth or yield of the plants used.

SUMMARY

A comparison was made of the growth response of wheat roots in the presence of nine different antibiotics dissolved in distilled water, tap water,

or nutrient solution. Bacitracin, polymyxin, potassium penicillin G, streptomycin tri hydrochloride, Terramycin hydrochloride, and thiolutin enhanced the growth at some concentrations when distilled water was used, though the percentage increase varied with the antibiotic. One-week-old Terramycin solutions were more beneficial than fresh solutions. The addition of these antibiotics to either tap water or nutrient solution failed to increase the growth of wheat roots. On the other hand, elongation in tap water was increased by solutions of catenulin and Magnamycin. A third group of antibiotics, including sodium rimocidin, streptomycin sulfate, and crude Terramycin, affected wheat root growth equally in distilled or tap water.

A series of experiments in which distilled water from two sources, demineralized distilled water, and tap water were tested revealed that one lot of distilled water was very toxic to the wheat roots, while all of the others permitted good elongation. Terramycin hydrochloride at a given concentration permitted the same growth of wheat roots in all of these waters.

Some evidence of the chelating effect of Terramycin hydrochloride was demonstrated by the addition of iron and by comparison with the chelating agent, ethylenediaminetetracetic acid. The addition of calcium ions to the solution reduced the injury to wheat roots caused by 10 and 32 p.p.m. Terramycin hydrochloride.

In limited tests, there was no increase in germination, or survival and growth of seedlings as a result of presoaking seeds in antibiotics or adding antibiotics to the soil. Also, preliminary tests in which plants in the field were sprayed with solutions of Terramycin hydrochloride revealed no beneficial effect of the antibiotic. It is pointed out that these results apply only to the conditions of the present trials, since soil variations and climatic, chemical, and biological factors are known to affect antibiotic action.

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Note

EFFECT OF PRESOAKING ON DORMANCY IN SEEDS

LELA V. BARTON

The seeds of many temperate-zone plants, especially trees and shrubs, exhibit some type of dormancy. A general method used to prepare these seeds for germination is pretreatment in a moist medium at low temperature, a process known as stratification. Working with *Picea glauca*, *Picea mariana*, *Pinus strobus*, and *Larix laricina*, Rudolf (2) found that soaking seeds in distilled water at 41° F. for 7 or 14 days was as effective for overcoming dormancy as cold stratification for one to three months. This effect was also found for *Pinus banksiana* in later work (Rudolf, 3). Other workers (Johnson, 1; Toumey and Durland, 4) have reported very little if any effect on the germination of tree seeds from soaking in water at higher temperatures.

Over a period of years, several tests of the effect of presoaking on the after-ripening of seeds have been made in this laboratory, but no striking gains in germination percentages or time required for germination have been demonstrated. Some of the data are presented here.

Seeds of *Malus sylvestris*, *Pinus contorta*, *Pinus ponderosa*, and *Berberis thunbergii* were obtained from Herbst Bros., New York, N. Y. The tests were set up so that lots of 100 seeds each were soaked in different amounts of tap water. For *Malus* and *Berberis*, these quantities were 2, 4, and 10 ml.; for *Pinus contorta*, they were 1, 2, and 5 ml.; and for *Pinus ponderosa*, which was limited in quantity, they were 5 and 10 ml. The quantities of water were selected to furnish a limited amount, an amount sufficient for complete swelling, and an excess. Presoaking was at two temperatures, 5° and 20° C., for 1, 4, 7, and 14 days. The water was changed twice weekly in the cases of the 7- and 14-day periods for seeds with an excess of water. Some mold appeared on the seeds soaked at 20° C. Neither seeds nor water were sterilized. Samples of presoaked seeds and dry controls were germinated immediately and others were placed in moist granulated peat moss at 5° C. for 1, 2, 4, and 8 weeks for after-ripening the dormant embryos. Following this stratification period, the cultures were transferred to controlled higher temperatures for germination. *Malus* and *Berberis* were germinated at a daily alternating temperature of 20° to 30° C. where the cultures were held at 20° C. for 16 hours, and at 30° C. for 8 hours each day. *Pinus* spp. were germinated at a constant temperature of 20° C. It should be kept in mind that single lots of 100 seeds only were used.

Some of the results are presented in Table I. Here are shown germination percentages obtained from seeds presoaked at 5° C. only in the quantity of water which was practically all absorbed in each case. It is evident

that *Malus* seeds were more dormant than any of the others, for they failed to germinate without stratification. Occasional seedlings appeared in four of the cultures while they were still at 5° C., but these never amounted to more than 4 per cent. Germination after 4 weeks of stratification may indicate a slight hastening of after-ripening by presoaking for 14 days, but additional tests would be necessary to establish this point. With this last possible exception, presoaking the seeds neither hastened the after-ripening process nor increased germination.

TABLE I
EFFECT OF PRESOAKING AT 5° C. ON AFTER-RIPENING AND GERMINATION

Seed	Presoaked		Per cent germination after stratification at 5° C. for different periods (in weeks)				
	Ml. H ₂ O	Days	0	1	2	4	8
<i>Malus sylvestris</i>	4	0	0	1	2	7	52
		1	0	0	0	4	38*
		4	0	0	0	8	14
		7	0	0	2	6	39*
		14	0	2	3	19*	47*
<i>Pinus contorta</i>	2	0	22	43	47	89	90
		1	26	23	65	83	88
		4	38	41	56	80	76
		7	21	48	66	78	78
		14	40	60	65	73	71
<i>Pinus ponderosa</i>	5	0	57	64*	69	52	67*
		1	49	62	58	74*	65*
		4	57	50	64	68*	68*
		7	55	51	42	63*	68*
		14	58	57	63	63*	56*
<i>Berberis thunbergii</i>	4	0	55	59	55	65	—
		1	55	58	55	32*	—
		4	48	59	57	49*	—
		7	47	69	71	51*	—
		14	23	52	60	38*	—

* Germination began during stratification at 5° C.

The pine species were selected to represent two different degrees of dormancy, neither of which is as pronounced as that of *Malus*. It will be seen from Table I that both *P. contorta* and *P. ponderosa* seeds will germinate without any after-ripening at 5° C. However, *P. ponderosa* seeds were not benefited by stratification, while germination percentages of *P. contorta* were increased substantially by such treatment (from 22 to 90 per cent, for example, in the case of the dry seeds). In neither pine species was there any evidence of increased germination, with or without after-ripening, as a result of presoaking at 5° C.

Berberis seeds, like those of *Pinus ponderosa*, showed no beneficial effects of either presoaking or stratification at 5° C. Many seedlings (up

to 56 per cent) appeared in the peat moss at 5° C. from all presoaked lots before the end of the 4-week period of stratification. This, coupled with the same tendency in *Pinus ponderosa* and in *Malus*, may be an indication of the hastening of after-ripening, though this is not reflected in the germination behavior upon transfer to a higher temperature.

Presoaking at 20° C. gave the same general results as presoaking at 5° C., but there were some differences. For example, while none of the seeds of *Pinus contorta* germinated in peat moss at 5° C. after presoaking at 5° C., there were occasional seedlings here from seeds presoaked at 20° C. for 14 days. Seed of *P. ponderosa* presoaked for 7 to 14 days at 20° C. germinated most promptly even though the final percentage of germination was reduced by these treatments.

The injurious effect of an excess of water and a temperature of 20° C. for an extended time was demonstrated clearly for *Berberis* (Table II). Presoaking for 14 days, even in 2 ml. of water, reduced the germination to 19 per cent when the seeds were not stratified. Stratification of the same seeds at 5° C. for 4 weeks brought about 60 per cent germination, indicating that the initial low germination may have been due to a delay in germination or a dormancy imposed by the presoaking. Using 4 or 10 ml. of water for presoaking 100-seed samples injured the seeds so that they did not

TABLE II
GERMINATION OF BERBERIS THUNBERGII SEEDS AFTER 0 AND 4 WEEKS OF
STRATIFICATION AT 5° C. FOLLOWING PRESOAKING IN 2, 4, OR 10 ML.
WATER AT 20° C.

Days presoaked at 20° C.	Per cent germination					
	No stratification			4 Wks. stratification		
	2 Ml.	4 Ml.	10 Ml.	2 Ml.	4 Ml.	10 Ml.
1	50	52	56	51	34	49
7	35	40	47	56	45	36
14	19	10	12	60	17	6

recover during the after-ripening period (Table II). Since *Berberis* and *Pinus ponderosa* were the only seeds exhibiting such injury, and since both of them germinated quite well without any after-ripening, it is assumed that the more-rapidly metabolizing seed is susceptible to soaking injury.

Because a 1 per cent thiourea solution is known to break the dormancy of freshly-harvested lettuce seeds, and 0.2 per cent potassium nitrate is effective in bringing about the germination of other dormant seeds, especially some grasses, these solutions were used for presoaking all the seeds described above. Harmful effects were demonstrated in certain cases, but no beneficial effects were evident.

The speed of germination, as measured by the days required for half of the final number of seedlings to appear, was also unaffected by presoaking. Many more tests than were run here would be required to demonstrate any differences in the speed of germination attributable to presoaking. Hastening of germination was shown, however, for after-ripened as opposed to nonafter-ripened seeds, whether dry or presoaked seeds were used. Certainly, if one considers the extra time required for the presoaking, no gain, but rather a loss, in total time required for germination could be demonstrated.

In another series of tests, freshly-harvested seeds were presoaked for 48 hours at 25° C. and then after-ripened in moist granulated peat moss at 5° C., after which they were planted in soil in the greenhouse. Species included in this study were: *Aronia arbutifolia*, *Evonymus rotundifolia*, *Malus niedwetzkyana*, *Myrica carolinensis*, *Physocarpus opulifolius*, *Pinus resinosa*, and *Pinus strobus*. No hastening of after-ripening as a result of presoaking was noted in any of these cases with the possible exception of *Myrica*, which showed some advantage of presoaking after 12 weeks of stratification. This advantage was not so evident after 16 weeks at 5° C. In the case of *Aronia*, additional soaking periods of 1, 2, and 3 days at 5°, 10°, or 25° C. failed to affect dormancy.

It is concluded that under the conditions and for the seeds tried in these tests, presoaking does not hasten germination or after-ripening.

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KINETICS OF INSECTICIDAL ACTION BASED ON THE PHOTOMIGRATION OF LARVAE OF *AEDES AEGYPTI* (L.)

H. P. BURCHFIELD AND ELEANOR E. STORRS¹

The activity of many organic insecticides can be measured through their ability to inhibit the photomigration of larvae of *Aedes aegypti* (L.) (1). This method has been used for the bioassay of plant extracts containing DDT, lindane, chlordan, heptachlor, etc. (1, 2, 5), and is generally useful for the determination of compounds which produce rapid muscular disorientation or paralysis of the test larvae.

It is based on measurements of the time required for a fixed response rather than on lethal dose; therefore comparative studies on the activities of various materials should provide useful information on the kinetics of insecticidal action.

Powers (9), Gersdorff (4), and others have studied the toxic action of inorganic salts on goldfish as a function of time and obtained evidence that the relation between concentration and the time required to kill is hyperbolic. Powers disregarded the extreme concentration ranges in his data and proposed a linear reciprocal curve for the central region. While this is satisfactory for bioassay work, it does not provide a comprehensive picture of the kinetic factors involved in toxic action. Burchfield, Hilchey, and Storrs (1) investigated the inactivation of mosquito larvae by DDT, methoxychlor, and lindane and proposed an equation with two limiting asymptotes which served to describe the relation between T_{50} (time for immobilization of 50 per cent of the larvae) and concentration over the entire range studied.

This present work extends these studies into higher concentration ranges and compares the inactivation rate constants of a wide variety of compounds with insecticidal activity.

Evaluation of these constants is unnecessary in routine bioassay since satisfactory results can be obtained from logarithmic-reciprocal plots of the central portions of the curves. However, the relation between dose and time of response is probably a general one, not necessarily limited to any particular species or group of chemicals. For this reason a comparative study of a series of highly active insecticides was made to illustrate the differences between them and establish the fundamental nature of the regression curve.

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MATERIALS AND METHODS

The relationship between concentration of insecticide and T₅₀ was measured over the complete range of activity of the following materials: allethrin, lindane (γ isomer of 1,2,3,4,5,6-hexachlorocyclohexane), methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane], parathion (*O,O*-diethyl *O-p*-nitrophenyl thiophosphate), Dilan [a mixture of 2-nitro-1,1-bis(*p*-chlorophenyl) propane and 2-nitro-1,1-bis(*p*-chlorophenyl)-butane], Rhothane [2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane], dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene), DDT [2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane], heptachlor (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-*endo*-methanoindene), chlordan (1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene and related compounds), Strobane (chlorination product of terpene hydrocarbons containing *ca.* 66 per cent Cl), aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene), and toxaphene (a chlorinated camphene corresponding approximately to C₁₀H₁₀Cl₈).

T₅₀ measurements were obtained at 10, 1.0, and 0.1 p.p.m. on 2,4-dinitro-6-cyclohexylphenol, Diazinon (*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) thiophosphate], 2,2-bis(*p*-ethoxyphenyl)-1,1,1-trichloroethane, piperonyl butoxide [α -[2-(2-*n*-butoxyethoxy) ethoxy]-4,5-methylenedioxy-2-propyltoluene], Chlorobenzilate (ethyl *p,p'*-dichlorobenzilate), and sodium cyanide. Piperine, Thanite (isobornyl thiocynoacetate), rotenone, nicotine, copper sulfate, and sodium arsenite were tested but failed to inactivate 50 per cent of the larvae within three hours.

The organic acaricides and insecticides were dissolved in Carbitol [2-(2-ethoxyethoxy)ethanol] at concentrations of 1000 p.p.m. and serially diluted with solvent to give solutions within the desired range. One ml. of each solution was then added with swirling to 99 ml. of water containing 100 larvae of *Aedes aegypti* and 1.0 p.p.m. Pluronic F-68 (a polyethylene-polypropylene glycol) as a conditioning agent. Concentrations in the final test solutions ranged from 10 p.p.m. down to 0.002 p.p.m. for the most active compounds. The number of concentrations used for each material that was evaluated in detail varied from 15 to 7, depending on the range of activity. The stock solutions of materials such as nicotine and sodium cyanide were prepared in water and diluted similarly except here only a few concentrations were employed.

Immediately after preparation the suspensions containing the insecticides and larvae were transferred to a migration chamber (1), and after the onset of paralysis the percentage of larvae immobilized was determined at two- to three-minute intervals. T₅₀ values were then interpolated from graphs of time against per cent inhibition and converted back to a stand-

ard reference point by subtracting 0.7 minute from the observed values for each hour after removal of the larvae from the nutrient solution (2).

In order to minimize fluctuations in larval resistance as much as possible the entire dilution series for each compound was tested on the same day using larvae drawn from the same batch. Each material was retested at least once on different days to insure that the data presented were typical. Heptachlor at 0.1 p.p.m. was included in each series of tests as a reference standard.

Second instar larvae of *Aedes aegypti* reared under the conditions previously described (2) were used throughout the entire series of experiments.

EXPERIMENTAL RESULTS

Most of the materials known to be active insecticides produced rapid immobilization of the larvae in the T₅₀ test. Since a large number of readings were made on each compound and the dilution series were necessarily irregular, only the data at 10, 1, 0.1, and 0.01 p.p.m. are presented (Table I). These are arranged in ascending order of the T₅₀ at the highest concentration. At 10 p.p.m. the time required for 50 per cent immobilization of

TABLE I
T₅₀ IN MINUTES AT FOUR CONCENTRATIONS OF VARIOUS INSECTICIDES

Insecticide	T ₅₀ at concentration of insecticide				Optimum range for bioassay (p.p.m.)	T ₅₀ of standard*
	10	1	0.1	0.01		
Allethrin	<1	<2	43	**	1.0-0.1	28.6
Lindane	<3	7.5	25.4	182	0.2-0.01	25.5
Methoxychlor	4	4	31.4	**	0.2-0.08	25.6
Dilan	4.5	6.5	44.1	**	0.5-0.05	30.6
Parathion	7.8	19.0	50	**	0.2-0.02	30.7
DDT	8.3	11.6	18.4	116	0.1-0.005	30.3
Rhothane	10.5	13.5	63.7	**	0.2-0.02	33.2
Dieldrin	13.8	15.5	40.5	137	0.2-0.005	30.7
Heptachlor	15.5	16.0	29.5	93.5	0.1-0.005	29.5
Chlordan	16.7	22.1	47.2	170	0.2-0.01	25.4
Stroban	23.3	40.1	171	**	2.0-0.1	29.6
Aldrin	26.6	31.3	45.4	148	0.2-0.005	28.9
Toxaphene	36.5	46.2	144	**	1.0-0.05	29.9

* 0.1 p.p.m. heptachlor.

** T₅₀ >300 minutes.

the test larvae varied from the almost instantaneous action of allethrin to 36.5 minutes for toxaphene. At 1.0 p.p.m. a few significant increases in T₅₀ and minor inversions in order occurred, but for most compounds it would be difficult to distinguish between different concentrations in this range. At 0.1 p.p.m. the situation changed drastically. All of the T₅₀ values were significantly higher and many inversions in order occurred. Allethrin,

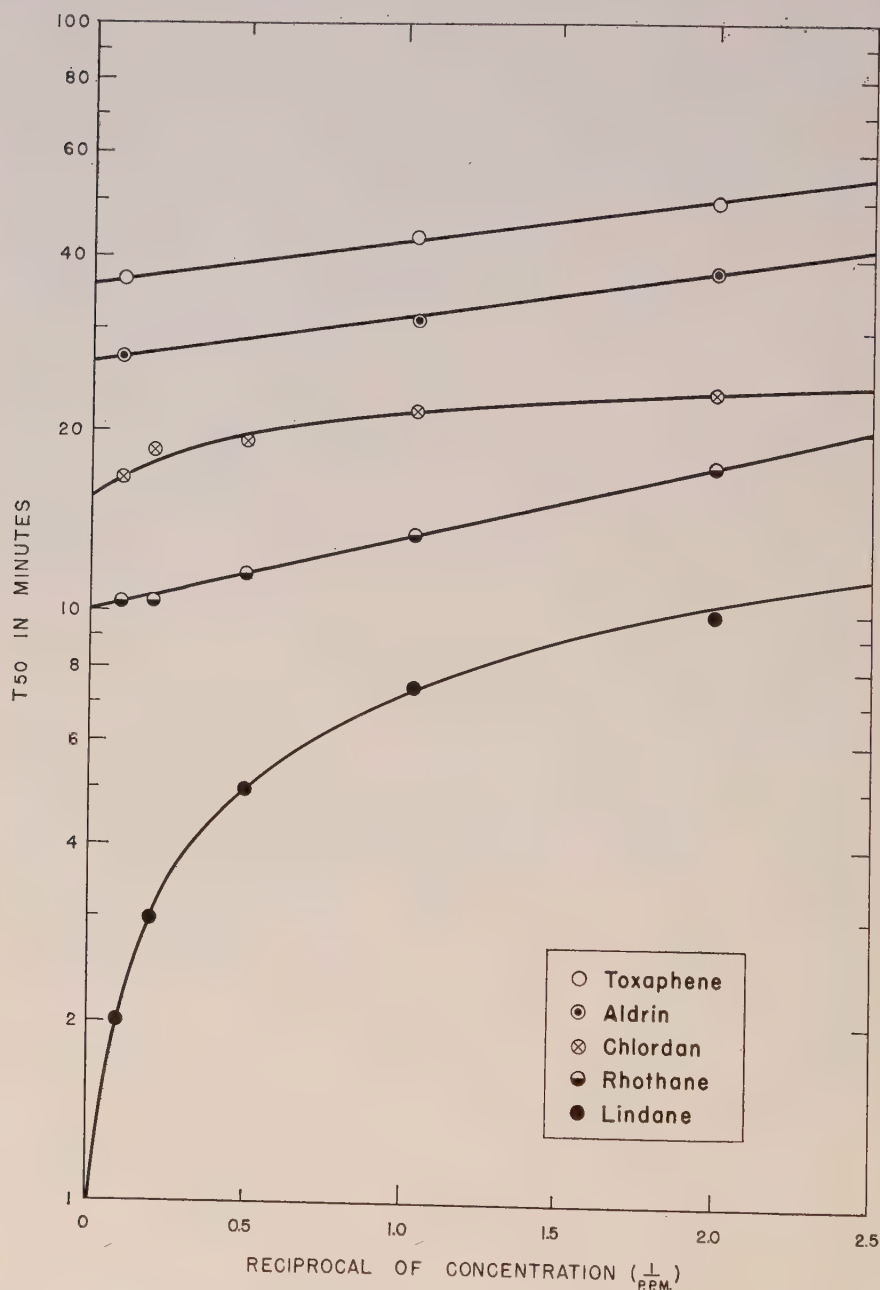


FIGURE 1. Estimation of minimum inactivation time (τ) for several insecticides by extrapolation of data at high concentrations.

which was the most active compound at 10 p.p.m., was only slightly faster than aldrin which was originally next to the slowest. However, T₅₀ values of an hour or less were obtained on all the materials in this group with the exceptions of toxaphene and Strobane. At 0.01 p.p.m. seven of the thirteen compounds failed to produce 50 per cent immobilization within five hours. These included some of the faster acting materials such as methoxychlor and Dilan as well as Strobane and toxaphene. Rhothane which had intermediate activity at 10 p.p.m. also failed to inactivate.

The results obtained on each compound were correlated over the entire range of activity by evaluating the constants in the regression equation described previously (1) which relates T₅₀ to the initial concentration of insecticide.

$$T_{50} = \sqrt{\tau^2 + \frac{A}{(c - c_0)^n}} \quad (1)$$

where τ is the minimum time required to inactivate 50 per cent of the larvae at infinite concentration, c_0 is the minimum concentration at which 50 per cent inactivation can take place regardless of the duration of the experiment, n is the slope of the line obtained by plotting $\log (T_{50}^2 - \tau^2)$ against $\log (c - c_0)$, and A is a position constant. It should be noted that the slopes of the lines are negative while n is positive in the form given in equation (1).

Approximate values for τ were determined for each insecticide by extrapolation. The reciprocal of the concentration was plotted against the logarithm of the T₅₀ at the four or five highest concentrations and a curve drawn which intercepted on the time axis (Fig. 1). A rough estimate for the probable value of c_0 was then made and provisional values for $\log (T_{50}^2 - \tau^2)$ and $\log (c - c_0)$ were calculated and plotted on rectangular coordinates. The provisional values were then adjusted until the relation was approximately linear. The slope of the line and the position constant were estimated by the usual methods. In general the preliminary estimates for τ were very much more satisfactory than the corresponding estimations for c_0 .

This procedure allowed for a direct comparison of data obtained over concentration ranges which differed widely. In all cases the correlation between observed and calculated values was 0.99+, indicating that the regression constants provide a good description of the behavior of each insecticide against the specific larval type used in the tests.

When listed in ascending order of τ (Table II) the sequence was approximately the same as the order of activity at 10 p.p.m. except for some minor inversion in the middle of the series where differences between the

TABLE II
TYPICAL REGRESSION CURVE CONSTANTS FOR VARIOUS INSECTICIDES
SATISFYING EQUATION (1)

Insecticide	τ (minutes)	c_0 (p.p.m.)	n	A	No. of observa- tions	T_{50} (in minutes) of standard*
Lindane	< 1	0.0045	1.05	56.2	12	25.5
Methoxychlor	3.5	0.0350	2.06	3.6	7	25.6
Parathion	5.0	0.0150	0.92	269	11	30.7
Dilan	6.4	0.0106	2.95	1.41	10	30.6
Rhothane	10.0	0.0310	1.31	81.3	9	33.2
Dieldrin	10.4	0.0006	1.04	138	14	30.7
DDT	11.2	0.0027	1.50	6.76	12	30.3
Heptachlor	14.5	0.0018	1.06	43.7	15	29.5
Chlordan	16.1	0.0028	0.97	25.4	11	25.4
Stroban	21.7	0.032	1.21	1122	7	29.6
Aldrin	29.6	0.0000	1.14	95.5	12	28.9
Toxaphene	35.6	0.0030	1.26	912	7	29.9

* Heptachlor at 0.1 p.p.m.

τ values were small. Allethrin was omitted since its effectiveness broke off too sharply on dilution to permit the calculation of satisfactory regression constants. This left lindane as the most rapidly acting material with a value for τ close to zero. The τ values then increased gradually throughout the series to a high of 35.6 minutes for toxaphene. It is evident that there was no connection between the speed with which an insecticide acted at high concentrations and the lowest dose which could produce 50 per cent immobilization.

Lindane, which acted very rapidly to begin with, was still effective at 0.0045 p.p.m., but methoxychlor which was almost as fast lost the ability to inactivate at a concentration eight times as high. Aldrin, on the other hand, had a high value for τ , and was so persistent on dilution that it could not be assigned a value for c_0 on the basis of the concentration range studied. In this case the regression curve was linear when $\log (T_{50}^2 - \tau^2)$ was plotted against $\log c$. The slopes of the regression lines (Fig. 2) tended to approximate unity, although there were some notable exceptions, particularly on Dilan and other compounds structurally related to DDT. The steepest slope was -2.95 obtained on Dilan and the shallowest was -0.92 obtained on parathion. Values for n and A showed no general tendency to correlate with one another or with τ and c_0 . It was interesting to note, however, that the DDT group which had the highest value for n also had the lowest value for A .

Although correlations within any series of tests carried out with the same batch of larvae were very good, agreement between the regression constants on experiments carried out with the same insecticides on different days was much less satisfactory (Table III). The T_{50} of the standard

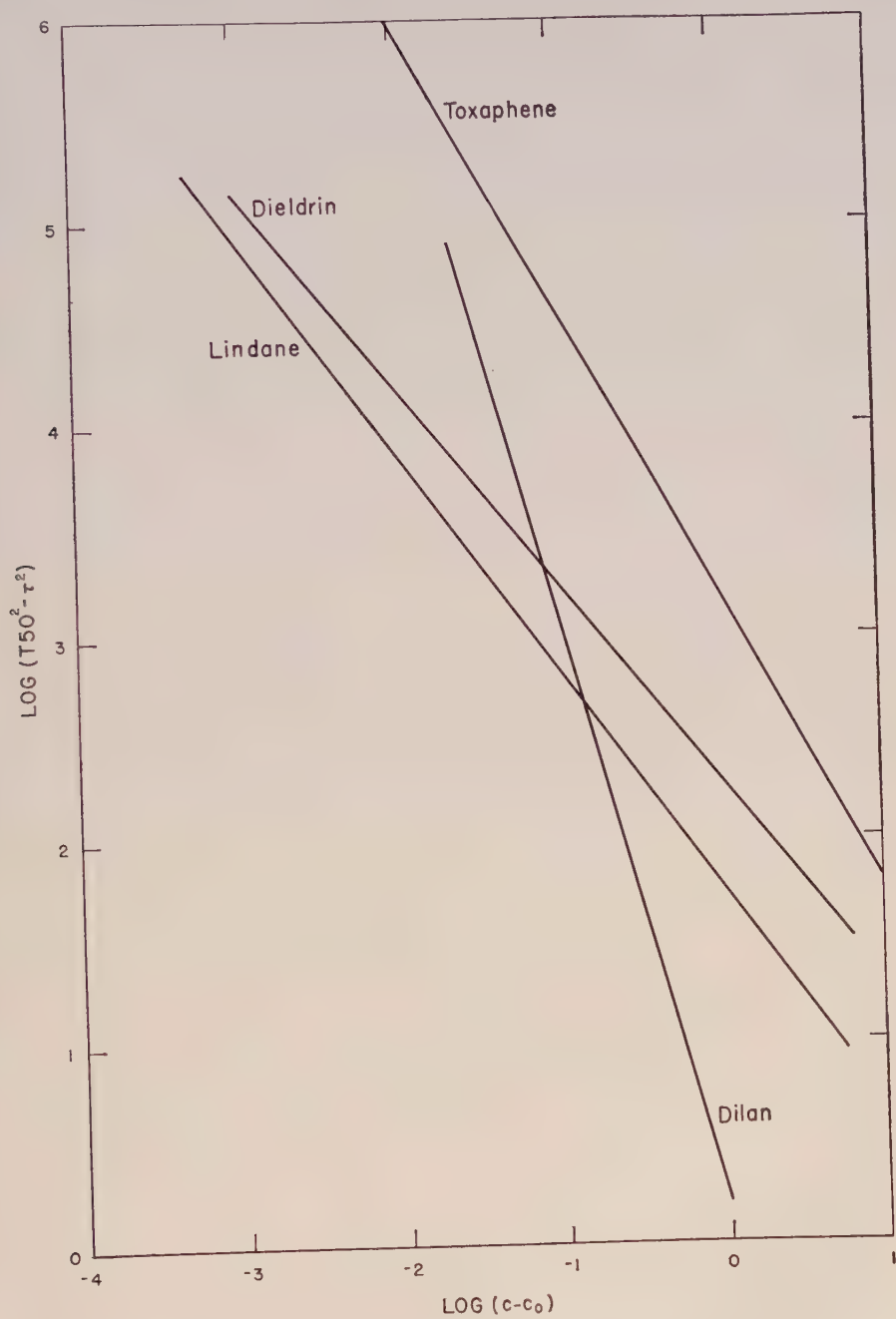


FIGURE 2. Linear regressions showing differences in slope for various insecticides.

TABLE III

REGRESSION CURVE CONSTANTS FOR HEPTACHLOR AND PARATHION SHOWING VARIATIONS OBTAINED ON DIFFERENT BATCHES OF LARVAE

Insecticide	Batch of larvae	Regression curve constant				
		τ	c_0	n	A	T_{50} (in minutes) of standard*
Heptachlor	1	14.5	0.0018	1.06	43.7	29.5
	2	15.0	0.0021	0.86	138	32.0
	3	14.9	0.0016	0.83	163	35.3
Parathion	4	1.0	0.041	0.75	447	27.0
	5	8.3	0.007	1.12	182	28.0
	6	5.0	0.015	0.92	269	30.7

* Heptachlor at 0.1 p.p.m.

(heptachlor at 0.1 p.p.m.) was 29.16 ± 2.44 minutes throughout the testing period which compared favorably with the average value found in earlier work (2). However, these variations led to large differences in the values of the regression constants.

In three sets of tests made on heptachlor, values for τ and c_0 agreed closely, n was reasonably constant, and A varied considerably. Results obtained on parathion were definitely less satisfactory. Some correspondence in replicate tests was obtained for τ and n , but values for A and c_0 differed widely. Reproducibility intermediate between these extremes was obtained on the remainder of the insecticides.

In addition to the insecticides already discussed, more limited data were obtained on a number of materials which were either less active in the photomigration test or unimportant economically (Table IV). 2,2-Bis-(*p*-ethoxyphenyl)-1,1,1-trichloroethane showed activity equivalent to methoxychlor while 2,4-dinitro-6-cyclohexylphenol and Diazinon were active

TABLE IV

RATES OF INACTIVATION OF MISCELLANEOUS TOXICANTS TO LARVAE OF *Aedes Aegypti*

Material	T_{50} (in minutes) at concn. (p.p.m.)		
	10.0	1.0	0.1
2,4-Dinitro-6-cyclohexylphenol	3.5	58	*
Diazinon	8.0	30	*
2,2-Bis(<i>p</i> -ethoxyphenyl)-1,1,1-trichloroethane	10	16	25
Piperonyl butoxide	16	*	*
Chlorobenzilate	41	*	*
Sodium cyanide	43	*	*

* $T_{50} > 300$ minutes.

at 1.0 p.p.m. but not at 0.1 p.p.m. Piperonyl butoxide, Chlorobenzilate and sodium cyanide were active at 10 p.p.m. but not at 1.0 p.p.m. Piperine, Thanite, rotenone, nicotine, copper sulfate, and sodium arsenite failed to inactivate 50 per cent of the larvae within three hours at 10 p.p.m. Evidently toxic symptoms induced by these latter compounds were long delayed and did not immediately affect the neural or muscular systems of the test larvae.

DISCUSSION

Data on the reproducibility of the regression constants indicate that the arrangement of the insecticides according to ascending order of τ (Table II) is arbitrary. A second series of experiments carried out under similar conditions would undoubtedly lead to a somewhat different arrangement, particularly in cases where the differences between values for τ are small. However, enough evidence has been accumulated to indicate that the general trend remains the same. Lindane acts rapidly and persists on dilution. Methoxychlor acts rapidly but loses effectiveness at comparatively high concentrations, while aldrin is slow but persistent. The biological material is not sufficiently uniform to give the regression constants the value of true constants, but nevertheless they serve to indicate trends which invite further experimental confirmation.

The tendency of the slopes of some of the regression lines to approximate unity is particularly interesting since it indicates that a comparatively simple rate law may be involved. Implicit differentiation of equation (1) with respect to concentration yields

$$\frac{dT_{50}}{dc} = - \frac{nA}{2T(c - c_0)^{n+1}} \quad (2)$$

or on simplifying the constants

$$\frac{dT_{50}}{dc} = - \frac{k}{T_{50}(c - c_0)^q} \quad (3)$$

In other words the change produced in the T_{50} by a very small change in concentration is inversely proportional to the T_{50} at the time of the change and inversely proportional to a power of the concentration in excess of the minimum amount required to produce inactivation.

The reproducibility of n in independent determinations made on different batches of larvae is certainly not better than ± 0.2 . Half of the insecticides tested gave values for n which approached unity within these limits (Table II) while two more, Strobane and toxaphene, were just outside these limits. Rhothane, DDT, methoxychlor, and Dilan gave results which were definitely greater than unity. It is interesting to note that these latter compounds are structural analogues. In view of this it is possible that for

many materials the rate of change of T_{50} with respect to concentration is inversely proportional to the *square* of the excess concentration. For some other materials it may be proportional to the cube or fourth power.

Regardless of the exact formulation of the equation there appears little doubt that the action of insecticides with respect to concentration and time is represented by a curve with two limiting asymptotes. The value for τ represents the minimum time for a chemical to be absorbed and translocated, and inactivate when the environment is saturated, and c_0 represents the minimum effective dose. It seems likely that any equation relating concentration to time must operate within these limits, and that the same type of relationship should hold for other physiologically active chemicals even when applied to different species.

In the earlier work (1) the existence of a limiting value for T_{50} was assumed, but data were not obtained at high enough concentrations to verify this. In these experiments T_{50} values were determined at high enough concentrations to show that τ has physical reality. Between 1 and 10 p.p.m. the log-reciprocal curves for many of the insecticides are linear within experimental error (Fig. 1). A few of them such as lindane and parathion are curvilinear, but these intercept on the time axis at values close to zero. It is evident then that for each insecticide there is a minimum time which must elapse before it can inhibit photomigration regardless of the initial concentration.

For each material there must also be a minimum concentration c_0 , below which 50 per cent immobilization cannot be obtained. However, this is more difficult to demonstrate experimentally since the data are more erratic at low concentrations and satisfactory extrapolations cannot be made. Ideally the value for c_0 should equal the LD_{50} obtained by the serial dilution technique (1), and from the limited data on hand this is approximately true for the chlorinated hydrocarbons (Table V). This interpreta-

TABLE V
COMPARISON OF LIMITING CONCENTRATION (c_0) WITH LD_{50} VALUES DETERMINED BY
SERIAL DILUTION AFTER A 24-HOUR INCUBATION PERIOD

Insecticide	c_0 (p.p.m.)	LD_{50} (p.p.m.)
DDT	0.0027	0.0035
Methoxychlor	0.0350	0.0233
Parathion	0.0150	0.0028

tion is not satisfactory for parathion, since here c_0 is 0.015 p.p.m. while the LD_{50} after 24 hours' incubation is *ca.* 0.003 p.p.m. or about one-fifth as great. These data are not strictly comparable since they were obtained by different experimental methods on larvae that presumably varied somewhat in resistance. However, in comparison with the results obtained

on DDT and methoxychlor it is obvious that parathion is not so effective in the T₅₀ test as it is in the serial dilution test. Presumably, during the 24-hour incubation period, secondary effects set in which are not detectable by photomigration experiments made during the first few hours after the addition of the toxicant. In any case the concept of a limiting concentration is somewhat arbitrary in working with biological materials, for the organisms themselves will undergo physiological changes during an indefinitely long period of incubation.

Equation (3) is intuitively satisfactory insofar as it relates rate of change of T₅₀ with respect to concentration to an inverse power of the excess concentration, but it is not clear why the rate should also be inversely proportional to the T₅₀ at the time of the change. The expression

$$\frac{dT_{50}}{dc} = - \frac{k_1}{(c - c_0)^p} \quad (4)$$

which on integration and combining constants leads to

$$T_{50} = \tau + \frac{B}{(c - c_0)^m} \quad (5)$$

is simpler in form and is more readily understandable. However, the sum of the differences between extrapolated and adjusted values for τ is +0.8 minute using equation (1) and -17.6 minutes for equation (5). There are more serious discrepancies in the values for c_0 since equation (5) requires

TABLE VI

COMPARISON OF VALUES FOR τ AND c_0 REQUIRED FOR EQUATIONS (1) AND (5)

Insecticide	τ			c_0	
	Extrapolated	Equation (1)	Equation (5)	Equation (1)	Equation (5)
Lindane	0	0	0	0.0045	0.0045
Methoxychlor	3.8	3.5	3.0	0.0350	0.0100
Dilan	3.9	6.4	5.8	0.0106	0.0000
Parathion	5.6	5.0	2.1	0.0150	0.0150
DDT	11.7	11.2	9.5	0.0027	0.0028
Rhothane	9.8	10.0	9.4	0.0310	0.0140
Dieldrin	13.8	10.4	6.9	0.0006	0.0000
Heptachlor	15.5	14.5	12.2	0.0018	0.0000
Chlordan	16.1	16.1	16.8	0.0028	0.0005
Strobane	21.2	21.7	22.7	0.0320	0.0083
Aldrin	26.0	29.6	23.4	0.0000	0.0000
Toxaphene	35.8	35.6	33.8	0.0030	0.0030

that this be zero for four of the insecticides (Table VI). This might be acceptable for aldrin where the dilution series was not run out far enough to estimate the limiting concentration, but it could not be true for Dilan or heptachlor. Dilan in particular breaks off sharply on dilution and a T₅₀

value cannot be obtained below 0.05 p.p.m. so obviously the interpretation provided by equation (5) is not in accordance with the experimental results.

The weight of evidences is probably insufficient to entirely eliminate equation (5) but until more accurate data become available the continued use of equation (1) to interpret the experimental regressions seems advisable. If sufficient information were available it might be possible to arrive at an analytic solution to the problem based on the laws of absorption and diffusion. Up to the present, attempts to do this have been unsuccessful, probably because of an incomplete knowledge of the sequence of processes involved between the initial absorption of the insecticide and the final inactivation of the test organisms.

Earlier attempts have been made to relate survival time to concentration. Warren (10) in his work with *Daphnia* called attention to the fact that the curves obtained on plotting time against concentration closely resembled equilateral hyperbolae. Ostwald (7, 8) applied a modified absorption formula while Carpenter (3), and Marcovitch (6) used the equation

$$K = \frac{1}{t} \log \frac{1}{c} \quad (6)$$

where t is the survival time, c is the concentration of chemical, and K is a constant. Since neither of these equations provides for limiting asymptotes it is evident that they will not adequately explain the data over the entire range of activity.

Powers (9) extensively reviewed the literature on velocity of fatality and on the basis of his own work proposed the relation

$$T = \sqrt{\frac{\tan \theta}{a}} \quad (7)$$

to define toxicity.

In this equation $\tan \theta$ is the slope of the line obtained by plotting the reciprocal of time against concentration and a is the intercept on the concentration axis. The experimental data depart from the line at both extremes, and even in the central region the relation is not truly linear. When applied to the type of data obtained in this present work the approximations for a and $\tan \theta$ were unsatisfactory. Furthermore, the theoretical threshold of toxicity (a), and the rate at which the action occurs ($\tan \theta$) are entirely independent of one another, so there is little to be gained by first extracting the constants from the data and then submerging their identities in a quotient.

All that the calculated value for toxicity (T) will tell is that the threshold toxicity and the slope of the line are in a certain ratio to one another. Actually there is an infinite number of combinations which will satisfy each finite value for T so all information on the magnitude of the effects is lost.

There is little prospect that a single constant can be found that will describe adequately both the ultimate toxicity and velocity of action of a physiologically active chemical. For the present it seems best to use a regression equation which fits the data over the entire range of activity and preserve the constants in their original forms.

In addition to providing information on the velocity of insecticidal action these data are also useful for assessing the range of usefulness of the T₅₀ test when applied to the bioassay of insecticide residues. With the exception of Strobane and toxaphene, all of the materials tested that were highly active had T₅₀ values of *ca.* 60 minutes or less at 0.1 p.p.m. and some of them could be detected at concentrations as low as 0.002.

The optimum analytical ranges (Table I) vary considerably. Thus allethrin is best tested at 1.0 to 0.1 p.p.m., lindane at 0.2 to 0.01 p.p.m., methoxychlor at 0.2 to 0.08 p.p.m., and toxaphene at 1.0 to 0.05 p.p.m. Obviously it is necessary to have some advance knowledge of the nature of the material to obtain quantitative results. However, if a T₅₀ value considerably greater than 60 minutes is obtained on a sample prepared at the rate of 100 g. of the crop per 100 ml. of test solution it can be assumed the more active insecticides are not present in concentrations greater than 0.1 p.p.m. and none of them are present in amounts greater than 1.0 p.p.m. This, of course, excludes rotenone, nicotine, and other materials inactive in the test, and is based on the assumption that interfering materials which might mask toxicity have been satisfactorily removed.

In some cases it might be possible to obtain information on the nature of an unknown material by making T₅₀ measurements on a series of dilutions. A material which is highly active at the original concentration but loses all activity on diluting 1:9 might very well be allethrin, methoxychlor, or Dilan, but is unlikely to be lindane or heptachlor. A determination of the slope of the regression line might be useful in a few specific cases since this value is independent of a knowledge of the absolute concentration of insecticide.

With some experience it is possible to guess at the nature of an insecticide by the response of the larvae during photomigration. Larvae treated with DDT contract spasmodically on exposure to the migration light but appear unable to move away from it. When treated with parathion they react sluggishly and move away from the source at a reduced rate, but in advanced stages may even move toward it. These observations are qualitative but may be useful in obtaining some knowledge of the nature of the insecticide in the hands of an experienced observer.

SUMMARY

The time required for immobilization of 50 per cent of a population of larvae of *Aedes aegypti* (L.) was determined in concentration series for

lindane, methoxychlor, Dilan, parathion, DDT, Rhothane, dieldrin, heptachlor, chlordan, Strobane, aldrin, and toxaphene. In all cases the relationship between time and concentration was represented by a curve with asymptotes corresponding to the minimum time required to produce immobilization at infinite concentration (τ) and the minimum concentration which could inactivate 50 per cent of the larvae in an indefinitely long reaction period (c_0). Value for τ varied from less than one minute for lindane to 35.6 minutes for toxaphene. Values for c_0 corresponded in some cases to LD₅₀'s obtained by the serial dilution method after 24 hours' incubation. These data lead to the provisional conclusion that the rate of change in T₅₀ with respect to concentration is inversely proportional to the T₅₀ at the time of the change and inversely proportional to a power of the concentration in excess of c_0 . For many insecticides the exponent is approximately 2, while for DDT and some of its analogues it may be 3 to 4. Bioassay results can be obtained on all of these materials in an hour or less at concentrations of 0.1 to 1.0 p.p.m. More limited data are given for a number of other materials which are less important economically or which failed to inactivate the larvae within a reasonable time limit.

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HISTOLOGICAL AND CYTOLOGICAL CHANGES INDUCED IN PLANTS BY CUCUMBER MOSAIC VIRUS (MARMOR CUCUMERIS H.)^{1,2}

CLARK A. PORTER³

Cucumber mosaic virus may cause formative effects and leaf distortion on solanaceous plants but is primarily known for its ability to cause green and yellow mottling on cucumber and other cucurbits. Necrotic areas of varying size may develop on the leaves of tulips and the leaves and stems of broad bean, while necrotic flecks are produced on Easter lily leaves by the fleck complex. The histological changes associated with these various macroscopic symptoms have not been described in detail even though they are critically important to a complete understanding of disease development.

Changes in the mesophyll of mottled cucurbit leaves were studied by Doolittle (7) who found the palisade cells of infected green areas were more closely crowded, longer and narrower than those of healthy leaves. Comparable cells in the yellow areas were more nearly isodiametric than in healthy leaves; spongy parenchyma was more compact and the plastids were smaller.

Such histological features of infected tissues might be valuable in identifying the various strains of cucumber mosaic virus and in distinguishing them from other viruses in cucumber (*Cucumis sativus* L.), tulip (*Tulipa gesneriana* L. var. Clara Butt), broad bean (*Vicia faba* L.), and Easter lily (*Lilium longiflorum* Thunb. var. Croft).

Detailed histological studies were made on naturally infected and inoculated plants growing in greenhouses and fields in Oregon to define the progressive changes induced by the disease and provide a diagnostic record of the microscopic symptoms. Results of these observations are presented in this paper.

MATERIALS AND METHODS

The cucumber mosaic virus used for inoculations in these studies was obtained from naturally infected cucumber plants in commercial fields in

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Oregon. It was identified by protection test in zinnia against the indicator strain of cucumber mosaic virus, *Marmor cucumeris* var. *judicis* H. All inoculations, except those on tulip, were made by extracting juice from infected cucumber plants and rubbing it onto the foliage by the carborundum method of Rawlins and Tompkins (18). Inoculations were made on the cotyledons and young leaves of cucumber plants, on the completely unfurled leaves of 6-inch broad bean plants, and on the youngest leaves of 5-inch Easter lily plants. Tulips were inoculated by hypodermic injection beneath the leaf epidermis.

Tissues were collected for microscopic examination from inoculated plants of Easter lily, broad bean, and the cucumber varieties Chicago Pickling and Snow's Perfection grown in the Oregon State College greenhouse during 1951-1952, inoculated tulip plants grown in the field near Corvallis, Oregon and collected during 1951, and from naturally infected cucumbers of the varieties Snow's Perfection and Lemon collected in the field during 1951. The viruses in the naturally infected plants were identified by subsequent protection tests in zinnia against the indicator strain of cucumber mosaic virus.

Healthy control tissues were obtained from disease-free field plants, and from greenhouse plants which were either uninoculated, or inoculated with juice from healthy plants. All control materials were collected at the same time and handled in the same manner as comparable virus-diseased materials.

Infected and control tissues were examined both in the living state and after sectioning in paraffin. Living materials were examined as freehand sections mounted in tap water and as whole mounts prepared by a vacuum technique (14). Randolph's modified Navashin solution (13) was employed as the killing agent for materials to be sectioned in paraffin. A modified tertiary butyl alcohol schedule (13) was used for dehydration and infiltration. All materials were embedded in paraffin of melting point range of 56° to 58° C. and sectioned at 5 to 25 microns. Sections were stained with several modifications of a basic iron hematoxylin-safranin combination (13).

Microchemical tests for fats, lignin, and wound gum were carried out according to Rawlins (17). Sudan IV was used in the tests for fats and the Maule reaction was employed for lignin determination. Wound gum is here regarded, as by Hewitt (10), to be water insoluble, to color red when treated with phloroglucinol-hydrochloric acid, and to give a negative test for lignin with the Maule reaction.

RESULTS

CUCUMBER

The various external symptom patterns produced in cucumber plants by cucumber mosaic virus have been well described (7). Symptom expres-

sion, essentially the same for all varieties used in this study, was characterized by yellow mottling of the diseased younger leaves. The green areas of mottled leaves generally do not differ in color from healthy leaves. However, occasional leaves exhibit dark green areas. These areas are raised, appear wrinkled, and may occur as islands surrounded by yellow tissue. All conspicuously mottled leaves are greatly reduced in size.

The histological and cytological abnormalities of mosaic-diseased cucumber leaves are limited to the mesophyll of the yellow and dark green raised areas. The pathological changes in the yellow areas vary depending upon the age of the leaf at the time of virus entry. Leaves infected when young exhibit a series of pathological changes during differentiation, while leaves infected when older show only cellular modifications of a single type. The green areas of mosaic-diseased leaves do not differ histologically from healthy leaves.

The first histological effects apparent in the yellow areas of young infected leaves are fewer cell divisions and premature cell vacuolation in the mesophyll as compared with the same tissue in healthy leaves (Fig. 1 A and B). The development of intercellular spaces is first evident in the spongy mesophyll of the yellow areas adjacent to the lower epidermis, and may be apparent while comparable normal cells are still closely packed. Thus, during the early stages of differentiation there are more intercellular spaces in the yellow areas than in comparable green areas. Also, the vacuolation of all mesophyll cells in yellow areas is much more pronounced than in the mesophyll of healthy leaves. The individual cells of yellow areas, however, are the same size and shape as the corresponding cells of healthy leaves. As tissue differentiation continues, the development of intercellular spaces in the mesophyll of healthy leaves proceeds faster than in the mesophyll of yellow areas. This results in a reversal of the earlier difference in the amount of intercellular spaces. This difference is very apparent in mature leaves where the intercellular spaces of the palisade tissue in yellow areas are much smaller than those of comparable healthy leaves (Fig. 1 C and D). The cells in healthy leaves may be larger than those of yellow areas. However, this difference is not constant since the cells in many yellow areas are indistinguishable in size from the cells in noninfected leaves.

During the early stages of development the mesophyll of yellow areas appears more mature than the mesophyll of healthy leaves. Later, at the time when the mesophyll of healthy leaves has matured, the yellow areas of infected leaves appear to be underdeveloped. This series of changes results in the mesophyll of yellow areas initially appearing hyperplastic and, later, appearing hypoplastic. The dark green raised areas of mottled leaves result from hyperplasia in the mesophyll. In these areas the palisade cells are longer and the spongy parenchyma has more cells and more intercellular spaces than in comparable healthy leaves (Fig. 1 E and F). The com-

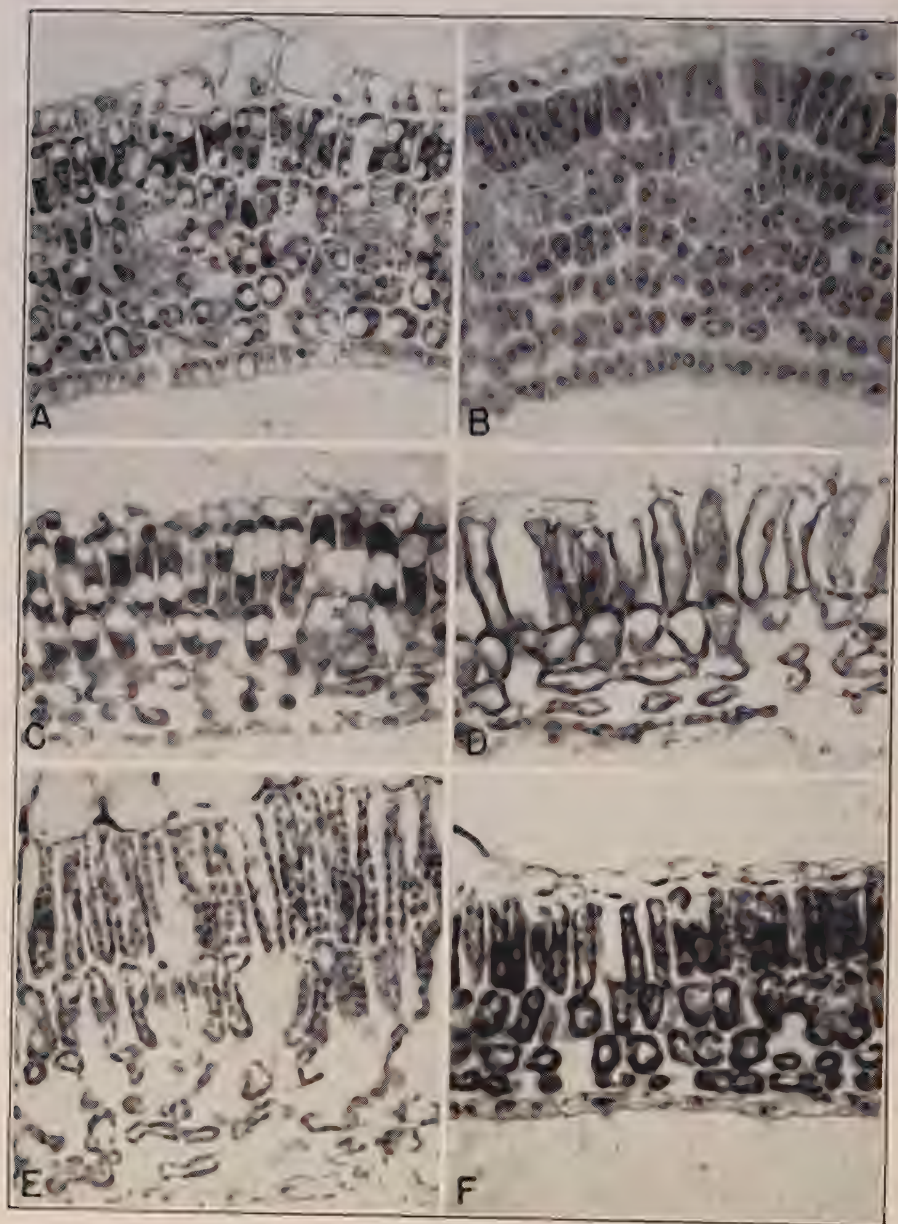


FIGURE 1. Cross sections of cucumber mosaic virus-infected and normal cucumber leaves. A, B, C, D, E, F ($\times 526$). (A) Premature cell vacuolation of mesophyll in yellow area. (B) Normal leaf same age as A. (C) Closely packed palisade cells of yellow area containing plastid aggregates. (D) Normal leaf same age as C. (E) Dark green raised area exhibiting abnormally long palisade cells, and spongy parenchyma with increased number of cells and intercellular spaces. (F) Normal leaf same age as E.

bination of these factors results in the dark green raised areas being thicker than corresponding areas in healthy leaves.

The plastids in yellow areas of the mesophyll in young leaves are yellow-green in color because their granules lack the definite green coloration of healthy plastids. At this early stage of differentiation the plastids are closely packed and angular in outline. In slightly older leaves, while the plastids in the mesophyll of healthy leaves are still closely packed and angular in outline, the plastids in the yellow areas assume circular shapes and are no longer closely packed. The plastids in the cells of the yellow areas are fewer in number and smaller in size than plastids in corresponding cells of healthy leaves. These differences are not so striking in smaller cells, but become more apparent as the plastids become separated by increase in cell volume. As differentiation continues, the plastids in yellow areas form aggregates, and may fuse within individual cells to form granular-appearing masses (Fig. 1 C).

Plastid aggregates also develop in the mesophyll cells of yellow areas when infection occurs during intermediate stages of leaf differentiation. These aggregates are similar in appearance to those developing in leaves infected when younger. There are no observable differences between the amount of intercellular spaces and length of palisade cells found in the later infected leaves and comparable healthy leaves.

EASTER LILY

The necrotic fleck condition of Croft Easter lily has been described by Brierley and Smith (3). The flecks initiate as chlorotic areas less than 1 mm. in diameter. These areas increase in size and become elongated parallel to the leaf venation. Increase in size is accompanied by the development of brown-colored lesions that often coalesce. Mature lesions, somewhat variable in size, are depressed below the leaf surface and the epidermis remains intact. The primary lesions which follow inoculation and the secondary lesions which result from systemic infection appear alike.

The lesions characteristic of necrotic fleck develop in the leaves of the Easter lily as a result of mesophyll degeneration. Necrosis begins in the bundle sheath cells, or in the adjacent mesophyll, and may initiate in any position about a vascular bundle (Fig. 2 A and B). The lesions are most often observed to originate centrally in the leaf and spread toward the upper and lower epidermis and in a lateral direction. All of the mesophyll about a vein will eventually become necrotic and collapse; until then the cells of the vascular tissues are not visibly affected.

In young leaves developing after systemic infection the initial symptom of cellular degeneration is the aggregation of plastids about the nucleus (Fig. 2 C and F). The plastids lose their distinct outlines and coalesce into a granular mass (Fig. 2 D). The nucleus remains visible for a time as a dis-

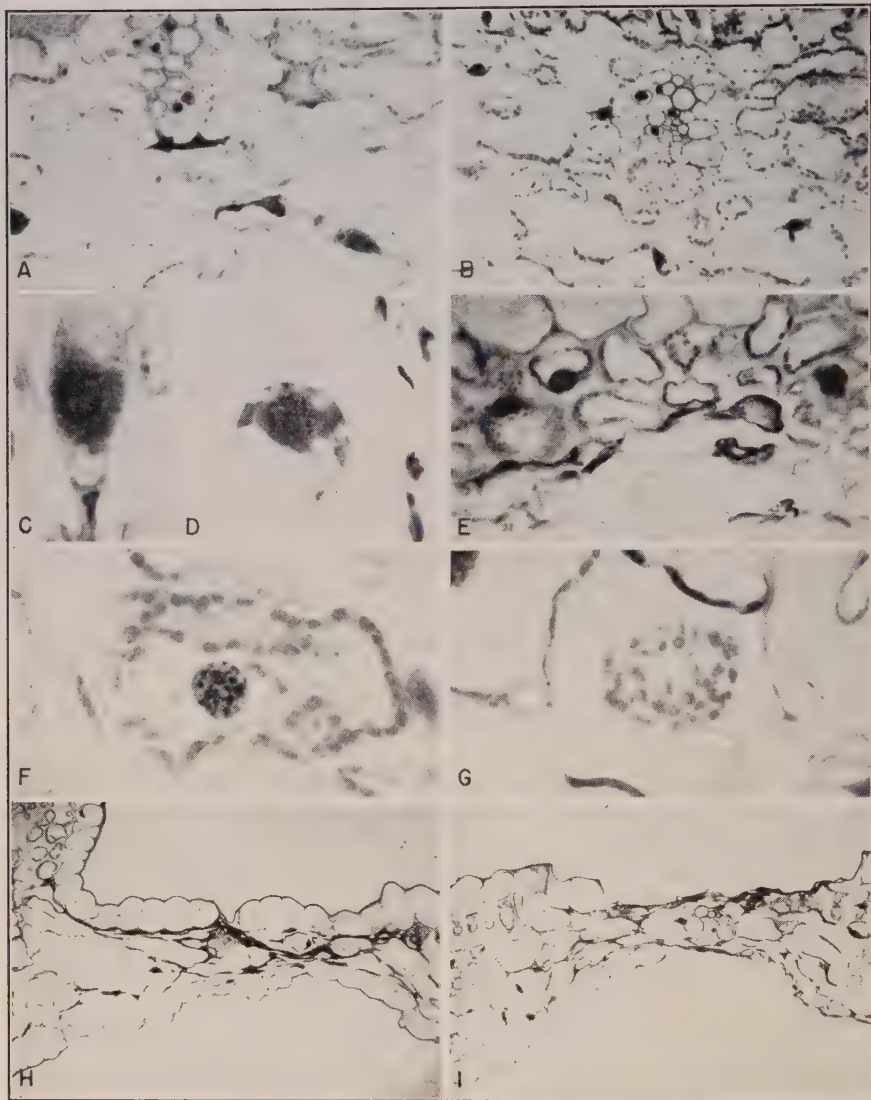


FIGURE 2. Cross sections of cucumber mosaic virus-infected and normal Easter lily leaves. A, B, E ($\times 255$); C, D, F, G ($\times 510$); H, I ($\times 64$). (A) Necrosis initiating in the bundle sheath and adjacent mesophyll. (B) Normal leaf. (C) Cellular degeneration beginning with aggregation of plastids about the nucleus in a mesophyll cell. (D) Plastids in degenerating cell have coalesced into a finely granular mass. (E) Dark-staining necrotic protoplasts within collapsing cells. (F) Normal mesophyll cell. (G) Vesiculated plastids in mesophyll cell of older leaf. (H) Epidermal collapse beginning over necrotic area. (I) Collapsed epidermal tissue forming intact cover over depressed lesion; initial necrotic effects apparent in vascular bundle.

tinct entity within the granular mass but soon loses its discrete appearance. All evidence of internal structure is lost and the nucleus becomes a homogeneous mass indistinguishable from the remainder of the protoplast. After the protoplast is no longer visibly granular it appears as dark-staining coagulated deposits closely appressed to the cell wall (Fig. 2 E). During the disintegration of the protoplast the cell walls begin to collapse and sometimes are nearly collapsed when the protoplast has reached its last stages of degeneration. In such cases the dark-staining deposits are pressed tightly between the converging walls. In other cells the contents degenerate and appear to be absorbed before the cell walls collapse. When the walls finally collapse, several may become closely aligned, often imparting the appearance of a single thickened wall.

When lesions develop in older leaves the plastids appear to degenerate in a slightly different manner. Plastids of affected cells become flattened in appearance and stain less intensely. They become vesiculated and then very diffuse in appearance (Fig. 2 G). Finally they lose their individuality and coalesce into a granular mass as in younger leaves.

In young leaves the epidermis is not visibly affected until after necrosis of all the mesophyll tissue in the fleck. As necrosis progresses and cells collapse, the lesion becomes a depressed area covered on both sides by normal-appearing epidermal tissue. Soon occasional epidermal cells become necrotic and collapse (Fig. 2 H). Eventually the entire epidermis covering a lesion may collapse but it remains as an intact cover (Fig. 2 I). The situation is different in older leaves where lesions may originate in the epidermis. In this case, the first visible necrosis occurs in one to several epidermal cells. Collapse of adjacent mesophyll cells in these older leaves may begin before there is much evidence of the necrotic processes described above.

Vascular tissues show no evidence of degeneration until after complete collapse of the mesophyll surrounding vascular bundles. In such late stages of mesophyll degeneration normal-appearing bundles may occur in the center of collapsed mesophyll (Fig. 2 I). Finally the vascular tissue also becomes necrotic and in very old lesions such tissue may be totally degenerated.

The histological symptoms characteristic of the fleck disease may be used to differentiate it from the rosette disease of Easter lily. The necrosis associated with fleck originates in the leaf mesophyll and is largely restricted to this tissue. In contrast, the necrosis associated with rosette is confined to the vascular elements of affected leaves (15).

TULIP

Brierley and Doolittle (2) describe the appearance of longitudinal gray streaks on the leaves of Clara Butt tulip one year after inoculation with

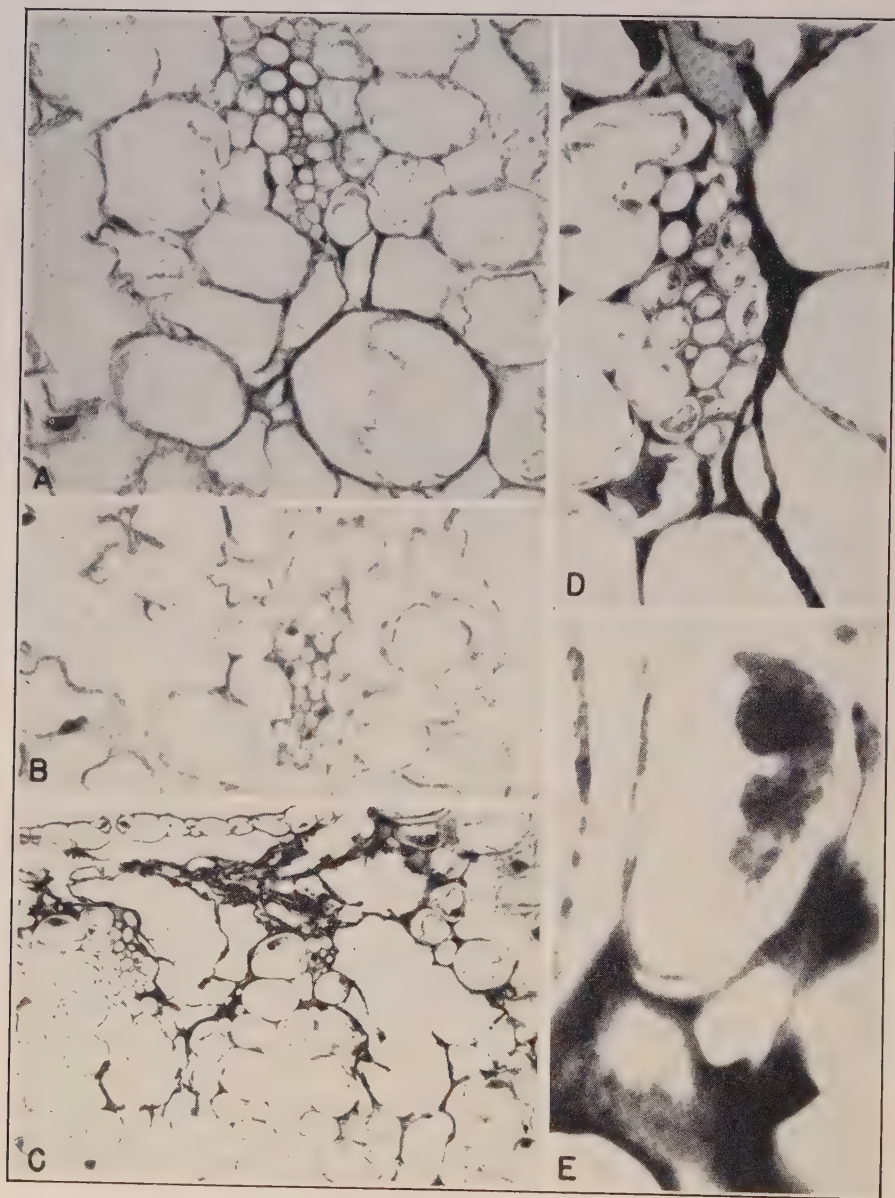


FIGURE 3. Cross sections of cucumber mosaic virus-infected and normal tulip leaves. A, B, C ($\times 100$); D ($\times 400$); E ($\times 800$). (A) Enlarged parenchymatous cells with thickened walls adjacent to bundle sheath. (B) Normal leaf. (C) Cell collapse resulting in wall aggregates interspersed with necrotic protoplasts. (D) Crushed bundle sheath cells accompanying cell enlargement and cell wall thickening. (E) Vesiculated plastids aggregated about nucleus.

celery and lily strains of cucumber mosaic virus. Somewhat similar symptoms are produced in Clara Butt tulip by Oregon isolates of cucumber mosaic virus. The initial symptom is the occurrence of small gray-green areas in the leaves. These areas increase in size, become chlorotic in appearance, and later yellowish-brown in color. Large areas of an individual leaf may ultimately be affected.

The first indication of degeneration in tulip leaves is an enlargement and wall thickening in the parenchymatous cells adjacent to bundle sheath cells (Fig. 3 A and B). These thickened walls are lignified as shown by the Maule reaction. This thickened wall condition is often exaggerated because of the deposition of the degenerating protoplast upon the inner surface of the walls. The enlargement of cells and thickening of cell walls is accompanied by crushing of some bundle sheath and parenchymatous cells (Fig. 3 D). Cell collapse is preceded by the development of thickened walls and degeneration of the protoplast.

The contents of collapsing cells appear as heavily stained coagulated masses. Complete collapse of several adjacent cells results in the occurrence of wall aggregates interspersed with dark-staining protoplasts (Fig. 3 C). During the early stages of symptom development the necrotic contents of collapsed cells appear to be largely absorbed. Cells affected in later development exhibit coagulated contents before there is evidence of collapse.

Cell collapse results in the formation of large voids within the mesophyll. These voids have their greatest dimension in a plane parallel to the long axis of the associated bundle and may extend from the upper to the lower epidermis as seen in a cross section of the leaf. Aggregated walls of collapsed cells form the boundaries between voids and the surrounding tissues. Coagulated cell contents may often be closely appressed to the void side of these boundaries.

Vascular tissues are initially unaffected. In advance stages of mesophyll degeneration bundles may show signs of necrosis. This condition does not prevail until the sheath is crushed and the surrounding mesophyll collapsed. Often, normal-appearing bundles are surrounded by crushed sheath cells and nearly encircled by voids. The only supports furnished such bundles are narrow plates of collapsed cells extending to the upper and lower epidermis.

Rarely does the epidermis show signs of degeneration. Small areas of epidermis may collapse over mesophyll that is in very late stages of degeneration. More often, the epidermis remains intact and turgid even in those areas where a void in the mesophyll occurs immediately beneath the epidermis (Fig. 3 C). For this reason, the affected areas of leaves are not depressed below the surface. The epidermis over such areas remains intact and maintains its normal position relative to the epidermis covering unaffected areas.

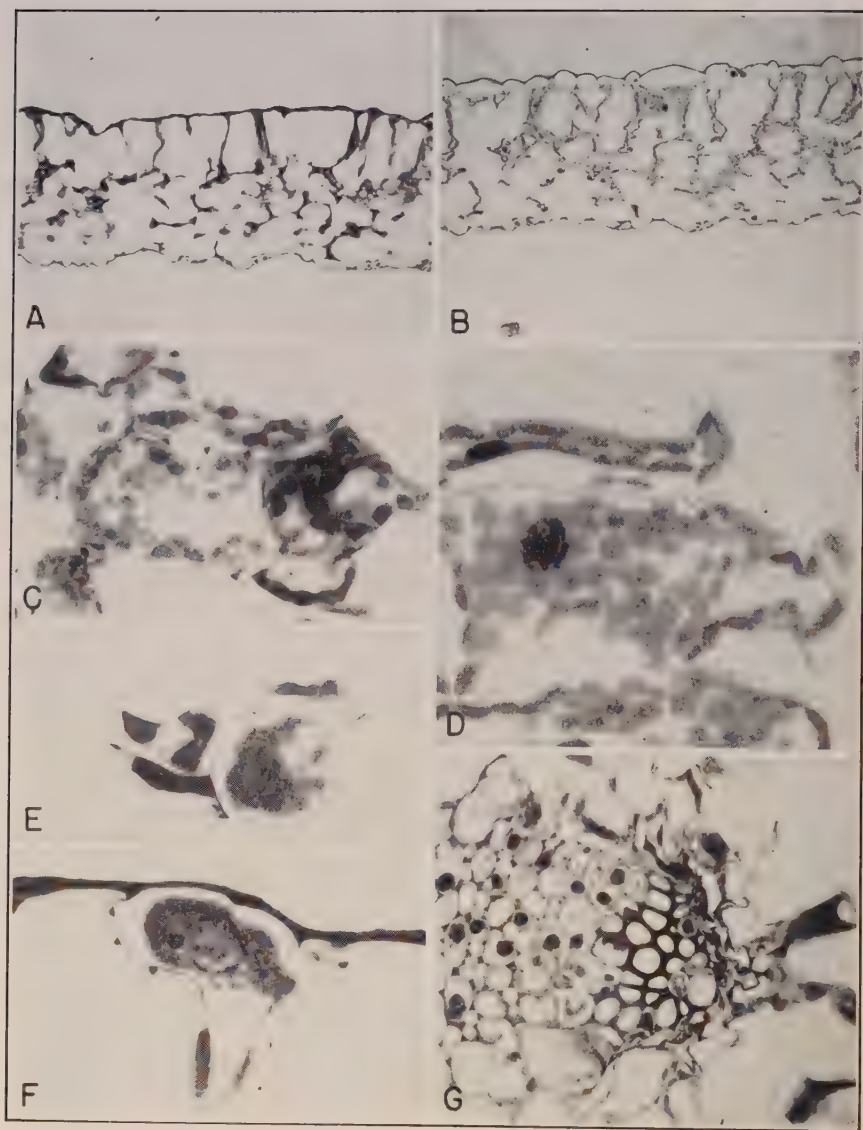


FIGURE 4. Cross sections of cucumber mosaic virus-infected and normal broad bean leaves. A, B ($\times 77$); C, D, E, F ($\times 620$); G ($\times 310$). (A) Necrotic lesion in inoculated leaf covered by collapsed epidermis. (B) Normal leaf. (C) Granular plastids aggregated about nucleus of mesophyll cell. (D) Normal mesophyll cell. (E) Vesiculate and diffuse plastids aggregated about nucleus of mesophyll cell. (F) Plastids fused into finely granular mass indistinguishable from cytoplasm. (G) Necrosis of bundle sheath adjacent to xylem of leaf bundle; exudate in xylem vessels to left of heavily lignified vessels.

After the initial enlargement of cells and the thickening of their walls, degeneration is evidenced by large deposits of necrotic material within uncollapsed cells. It appears that the protoplasts degenerate before collapse of the cells occurs.

Suitable materials were not available for a complete study of cellular degeneration. The effects, however, are first evident in the plastids and cytoplasm. The plastids of affected cells are often vesiculate and the cytoplasm is coagulated (Fig. 3 E). The nucleus remains discernible longer than other cell constituents but it eventually disintegrates and becomes indistinguishable in the mass of necrotic protoplasmic material.

BROAD BEAN

The initial symptom in broad bean infected with cucumber mosaic virus is the development of brownish-black, necrotic lesions at the site of inoculation. The lesions, at first circular and varying from 1 to 2 mm. in diameter, increase in size and become irregular in outline. The centers of the lesions become lighter brown in color and are depressed slightly below the leaf surface. Vein necrosis results when a lesion contacts a vein. Necrosis progresses along the vein to the midrib, continuing through the petiole and finally to the stem. Stem necrosis is first evident as a brownish-black, streaked discoloration along one side of the stem below the infected leaf. The necrosis develops both upward and downward in the stem but progresses downward more rapidly. There is also a lateral development of necrosis which may ultimately encircle the stem.

The lesions in broad bean leaves induced by inoculation with cucumber mosaic virus initiate in the palisade cells of the mesophyll. The protoplasts necrose and form deposits of dark-staining material confined within individual cells. The walls of such cells soon collapse. Cellular degeneration progresses downward and laterally through the mesophyll (Fig. 4 A and B). The necroses are equally apparent in the palisade and spongy mesophyll regions but decrease in severity laterally from the center toward the periphery of the lesions.

Cellular degeneration in the mesophyll is first evident in the plastids which become granular in appearance and tend to aggregate about the nucleus (Fig. 4 C and D). They soon become diffuse in appearance and sometimes become vesiculate (Fig. 4 E). At this time the dense cytoplasm becomes granular throughout. The plastids coalesce into finely granular masses which become indistinguishable from the cytoplasm (Fig. 4 F). The nucleus loses its normal appearance and becomes a dark-staining amorphous mass. The final stage in cellular degeneration is the collapse of the cell walls. Thus, the fully developed lesion consists of masses of necrotic protoplasmic material with collapsed cell walls dispersed throughout.

Degeneration of epidermal cells proceeds in a similar manner but does

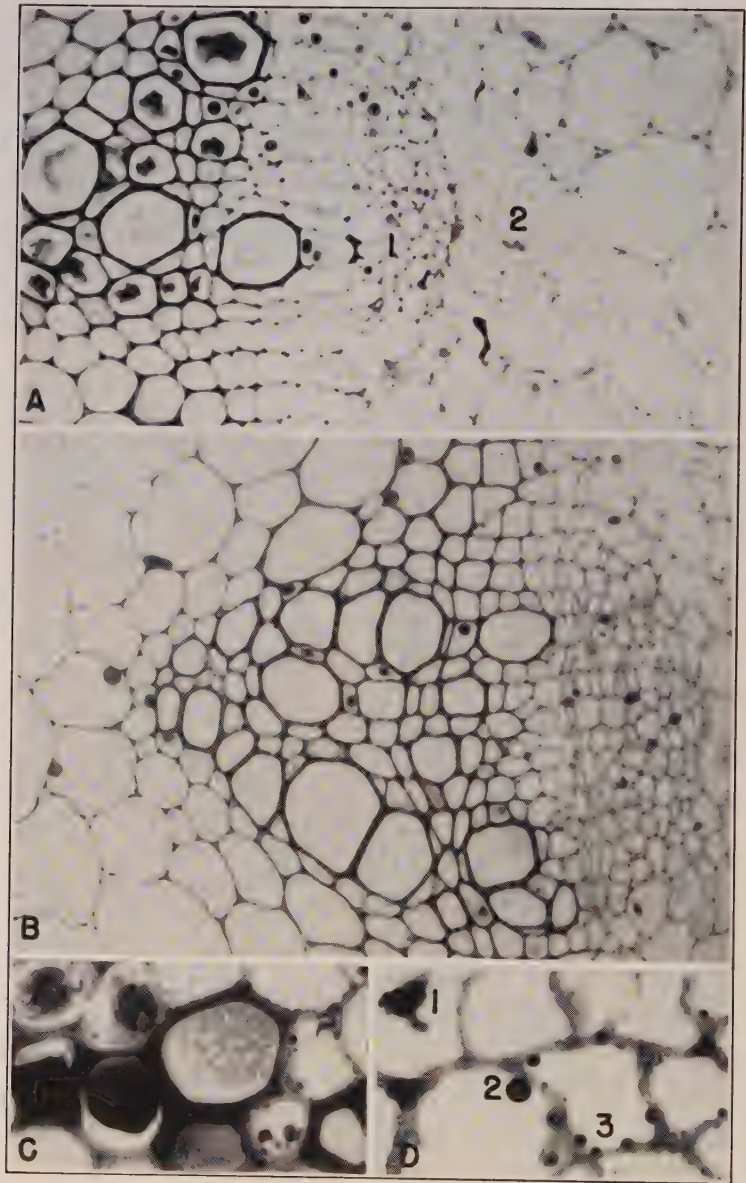


FIGURE 5. Cross sections of vascular bundles from cucumber mosaic virus-infected and normal broad bean stems. A, B ($\times 200$); C, D ($\times 800$). (A) Necrosis of phloem (1) and immature fibers at outer periphery of phloem (2); wound gum in vessels. (B) Normal bundle. (C) Finely granular exudate in large xylem vessel (center) and homogeneous exudate in smaller vessels (left.) (D) Spherical bodies in phloem; aggregate of spheres (1), single large sphere (2), and several small spheres (3).

not occur until after necrosis of the mesophyll beneath. The protoplasts of epidermal cells necrose but their walls collapse only over the central and most severely necrotic portion of the mesophyll. The radial, transverse, and inner tangential walls collapse but the outer tangential walls remain intact and stain heavily with safranin and hematoxylin. The collapse of cell walls results in a slight depression of the epidermis over the central portion of the lesion.

The sheath cells of vascular bundles necrose following degeneration of nearby mesophyll. The initial effects may occur in any portion of the sheath but most often originate in the region adjacent to the xylem (Fig. 4 G).

Changes in xylem vessel walls are evident in areas where the surrounding bundle sheath and mesophyll are necrotic. The walls of such vessels are stained much darker than those of normal vessels. This dark-staining effect is first evident at the corners of the vessels, subsequently extending around the entire wall as seen in transverse section. Simultaneously with changes in vessel walls, other cells within the bundle become necrotic until degeneration is general throughout the bundle.

An exudate is present in xylem vessels of those veins having necrotic sheath cells (Fig. 4 G). The staining reaction of the exudate with phloroglucinol-hydrochloric acid varies from reddish-yellow to very dark red. The exudate gives a negative Maule reaction for lignin and is water insoluble. This material is therefore considered to be wound gum. The differential reaction to phloroglucinol-hydrochloric acid may indicate that the wound gum is in various stages of formation.

Necrosis in the young stem is at first restricted to the phloem of a single vascular bundle (Fig. 5 A and B). The longitudinal progression of the necrosis through the stem is most rapid in or near the initially necrotic bundle. Later, the necrosis becomes rather general and involves also the cortex, cambium, xylem, and pith (Fig. 6 A). At a given level, the necrosis initially spreads from a necrotic bundle to adjacent bundles through the parenchyma of the cortex or pith. Necrosis also progresses longitudinally through various tissues other than those in or about the initially necrotic bundle.

Necrosis of the phloem involves sieve tubes, companion cells, parenchyma, and the fibers at the outer periphery of the phloem (Fig. 6 B). The most severe necrosis occurs in the phloem parenchyma adjacent to the fibers. In early stages of necrosis the cytoplasm of the parenchyma cells becomes granular in appearance. It soon forms deposits that line the cell walls, stain deeply, and often impart a thickened appearance to the walls. The walls collapse and the necrosed cells appear as masses of dark-staining, protoplasmic material interspersed with cell walls. Such masses are brownish-yellow in fresh sections and stain reddish-yellow with phloroglucinol-hydrochloric acid.

The first degenerative change apparent in the immature fibers at the

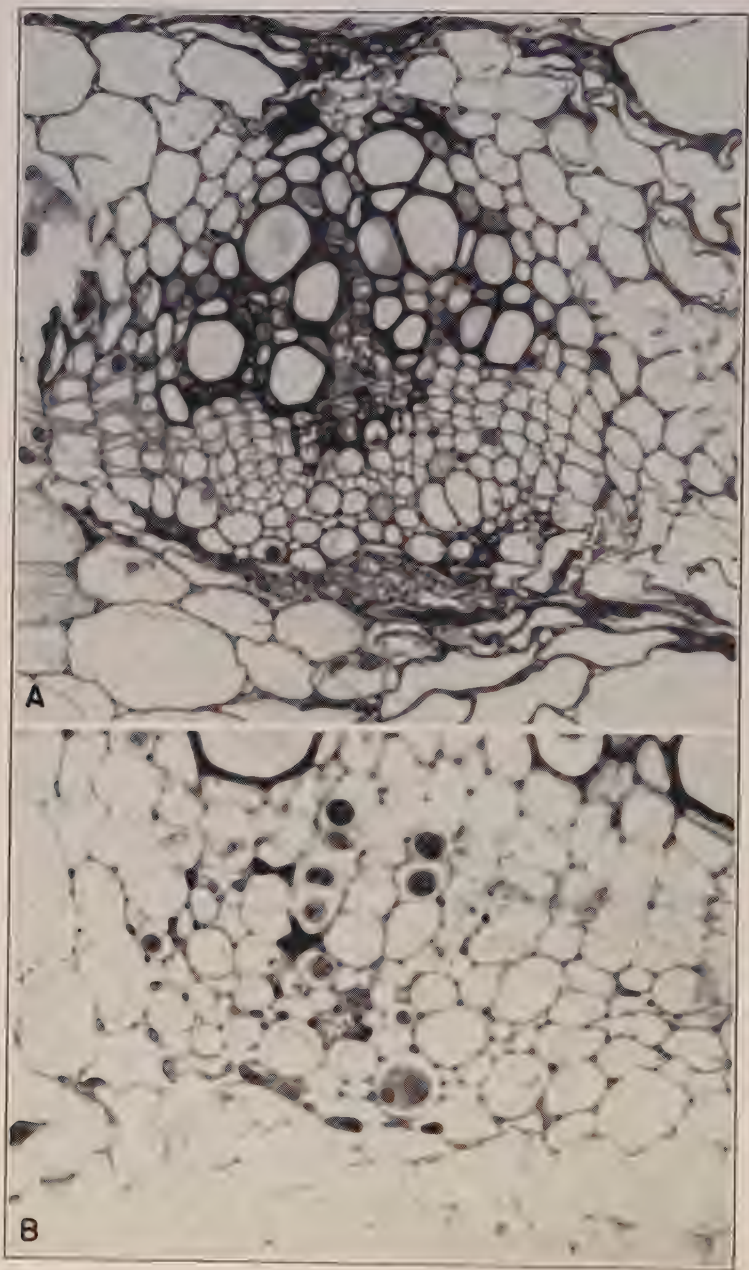


FIGURE 6. Cross sections of vascular bundles from cucumber mosaic virus-infected broad bean stem. A ($\times 200$); B ($\times 800$). (A) Necrosis general throughout vascular bundle and adjacent tissues. (B) Necrotic phloem.

phloem periphery is the distortion of cell walls. The protoplasts necrose and stain dark red with safranin. The fibers eventually collapse completely and the entire mass of degenerated fibers becomes continuous with the already necrotic phloem parenchyma.

Cortical parenchyma and fibers necrose in the same manner as the corresponding phloem cells but generally do not become affected until the necrosis is rather general throughout adjacent vascular tissue.

Both interfascicular and fascicular cambium may become necrotic. Isolated areas of interfascicular cambium may necrose or the necrosis may be continuous with that of the cortical parenchyma. Necrosis is usually restricted to tiers of cells wherein two or three primarily necrosed cells are apparent. The protoplasts become granular and degenerate into irregular masses within individual cells. Necrosis of fascicular cambium is apparent only in those regions where vascular tissues are severely necrotic, and is continuous with the degenerated vascular tissue. Large areas may be affected and form dark-staining masses which appear the same as those formed in necrotic phloem.

The first effect exhibited by the xylem is the deposition of wound gum in the vessels. Vessels may contain gum even though necrosis is not apparent in or about the bundle. The gum appears finely granular at first, and stains brownish-red with phloroglucinol-hydrochloric acid. Later the deposits become homogeneous and stain very dark red with the same reagent (Fig. 5 C). Necrosis of xylem parenchyma is first evidenced by protoplast degeneration followed by collapse of walls. In general, xylem necrosis is most severe near the cambium and decreases in severity deeper within the tissue. However, necrosis may appear first in a bundle in the primary xylem as a result of spread through pith parenchyma from a necrotic bundle. In such cases the primary xylem is the only portion of the bundle exhibiting necrosis and this occurs only in those regions of the stem where there are other bundles which are severely necrotic. In the most severe stages of necrosis the walls of vessels and parenchyma stain darker with safranin.

In regions of the stem where necrosis is most severe, degeneration of pith parenchyma is apparent. Such degeneration is usually in areas adjacent to the severely necrotic bundles, but may extend through the parenchyma to other bundles. Eventually the parenchyma cells of the pith collapse, forming necrotic masses like those described as occurring in phloem parenchyma. Isolated areas of necrotic pith parenchyma may be seen when the stem is viewed in cross section. Such necrotic areas result from the longitudinal spread of necrosis through the pith.

Spherical bodies ranging in size from less than 1 micron to 6 microns are present in phloem, cambium, and xylem parenchyma cells of severely necrotic stems (Fig. 5 D). Aggregates of bodies of intermediate size are usually found in cells adjacent to severely necrosed cells. These occur most

abundantly in the phloem. The individual bodies within an aggregate may be somewhat ovoid. The smaller bodies occur at greater distances from obvious necroses and usually appear to line the inner walls of phloem, cambium, and xylem parenchyma cells. Occasionally, large spheres are present in cells but they are not as general in occurrence as the intermediate and small-sized bodies. Nuclei may be present within the same cells where the various bodies occur. All of the bodies are colorless in unstained sections, stain light to bright pink with safranin, and do not stain with Sudan IV.

DISCUSSION

In general, the results of the present study of mosaic-diseased cucumber leaves agree with the studies of other mosaic diseases (4, 6, 7, 8, 9, 11, 12, 16, 19, 20). The histological and cytological abnormalities of mottled cucumber leaves are restricted to the mesophyll of the yellow and the dark green raised areas. The yellow areas in young leaves appear hyperplastic because they exhibit certain characteristics of older, healthy leaves. This early differentiation in yellow areas is attributable to the suppression of cell divisions, the premature cell vacuolation, and the early development of intercellular spaces. The yellow areas in old leaves appear hypoplastic because they do not complete the typical mesophyll differentiation of healthy leaves. The yellow areas have less intercellular spaces than normal and the palisade cells may be slightly shortened. However, the palisade cells of yellow areas are usually the same size as those of comparable healthy leaves. Some mosaic diseases inhibit the differentiation of mesophyll into palisade and spongy parenchyma regions (6, 8, 9, 16). This is not the case in mosaic-diseased cucumber leaves where development of distinct palisade and spongy parenchyma regions were observed to occur in all cases. Goldstein (9) has shown that leaves infected when young exhibit more marked internal abnormalities than leaves infected when they were partly differentiated. The results of the present study are in agreement with Goldstein's conclusions.

The dark green raised areas of mosaic-diseased cucumber leaves result from hyperplasia in the mesophyll. This is in agreement with Doolittle's findings concerning such areas (7). When compared with the mesophyll of healthy leaves, the palisade cells of dark green raised areas are decidedly longer, and there is more spongy parenchyma with a greater amount of intercellular spaces. These areas are thicker than comparable areas of healthy leaves.

The present study indicates that both inhibition of development and destruction of plastids occur in the yellow areas. Inhibition results in reduction in number and size of the plastids. Some plastids appear to develop normally for a time and then form aggregates. These aggregates may be formed in leaves during both the early and intermediate stages of dif-

ferentiation. This may be the initial stage in plastid degeneration since it seems that such plastids do not recover. This conclusion appears to be substantiated by the fact that the plastids in yellow areas of mosaic-diseased cucumber leaves have never been observed to return to a normal condition as has been reported to occur in mosaic-diseased sugar cane, canna, and tomato (5). Some plastids in yellow areas of cucumber leaves become diffuse and coalesce into granular-appearing masses. The vesiculation and coalescence of chloroplasts may be induced by agents other than viruses (8, 21, 22). Esau (8) does not regard plastid changes in mosaic plants as responses to any specific action of the viruses.

The necrotic symptoms produced by cucumber mosaic virus in tulip and broad bean and by the fleck complex in Easter lily originate in the leaf mesophyll. The necrosis in Easter lily and tulip is restricted primarily to this tissue, and invades vascular tissue only during the late stages of mesophyll degeneration. The resulting vascular degeneration appears to be not a direct result of virus infection but rather an indirect effect related to association with the surrounding dead tissues. Contrary to this, the necrosis of leaves and stems of broad bean rapidly becomes general throughout all tissues. In this host, it appears that degeneration of vascular tissue results from direct invasion by the virus.

The necrosis in tulip is associated with cell enlargement and cell wall thickening. Similar effects are not apparent in Easter lily and broad bean. The protoplasts in tulip and broad bean degenerate before cell collapse occurs while in Easter lily degeneration of the protoplast and collapse of the cell occur almost simultaneously. Spherical inclusions, somewhat similar to those found in broad bean, are reported to occur in sugar cane chlorotic streak (1).

SUMMARY

The histological and cytological changes induced by cucumber mosaic virus in cucumber leaves, tulip leaves, broad bean leaves and stems, and by the fleck complex in Easter lily leaves were observed during the course of disease progression. In all cases, the initial effects originate in the mesophyll of affected leaves. Symptoms are confined to this tissue in mottled cucumber leaves but the necroses produced in the other species ultimately involve other tissues.

Yellow areas in young cucumber leaves appear hyperplastic due to suppression of cell division, premature cell vacuolation, and early development of intercellular spaces in the mesophyll. In mature leaves, these same areas are hypoplastic because they do not complete the typical mesophyll differentiation of healthy leaves. The plastids in yellow areas are both inhibited in development, and destroyed. Inhibition results in reduction in number and size of the plastids. Plastid aggregates form in yellow areas

in leaves infected during early differentiation and also in leaves infected during later differentiation. The plastids in the aggregates may fuse into granular-appearing masses.

The dark green raised areas of mottled cucumber leaves result from hyperplasia in the mesophyll. In these areas the palisade cells are longer, and the spongy parenchyma has more cells and more intercellular spaces than in comparable healthy leaves.

Necrosis in Easter lily leaves is restricted primarily to the mesophyll and does not progress to the epidermal and vascular tissues until the mesophyll is degenerated. Cellular degeneration begins with the plastids aggregating about the nucleus. The plastids then coalesce into a granular mass and the nucleus loses its characteristic appearance. The entire protoplast degenerates into a homogeneous, coagulated mass deposited about the cell wall. The cells collapse almost simultaneously with protoplast degeneration.

Necrosis in tulip leaves is restricted primarily to the mesophyll and is associated with cell enlargement and cell wall thickening. Some crushing of bundle sheath and parenchyma cells occurs as a result of cell enlargement. Necrosis does not involve vascular tissue until late stages of mesophyll degeneration. Epidermal tissue rarely exhibits signs of necrosis but rather remains intact and turgid. Cellular degeneration is first evident in the plastids and cytoplasm. The cells collapse only after complete protoplast degeneration.

Necrosis in broad bean leaves spreads rapidly from the mesophyll to the epidermal and vascular tissues. The necrosis in the stem is at first restricted to the phloem of a single vascular bundle, but soon becomes general throughout the cortex, phloem, cambium, xylem, and pith. Protoplast degeneration appears to proceed much in the manner as described for Easter lily. However, as in tulip, the protoplast degenerates before cell collapse occurs. Spherical bodies occur in phloem, cambium, and xylem parenchyma cells of severely necrotic stems.

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STUDIES ON THE NATURE OF FUNGICIDAL ACTION.

III. EFFECTS OF FUNGICIDES ON POLYPHENOL OXIDASE IN VITRO

ROBERT G. OWENS

INTRODUCTION

The bactericidal and fungicidal activity of certain classes of chemicals has been attributed to their ability to inactivate catalytic heavy metals (1, 3, 13). Representative compounds of dithiocarbamates and 8-hydroxyquinolines have been shown to inactivate catalase and polyphenol oxidase also (7). This suggests that bound as well as free iron and copper are subject to attack by these compounds. Since iron and copper enzymes frequently occupy the terminal positions in the transfer of electrons to oxygen in respiration, they are of considerable interest as potential sites of fungicidal action.

In order to gain more information on the relative activity of metal complexing fungicides as inactivators of heavy metal enzymes, studies have been carried out with representative compounds chosen from the dithiocarbamates, bisdithiocarbamates, xanthates, thioureas, 8-hydroxyquinolines, and other classes of fungicides. The present paper deals with the effects of various members of these groups on polyphenol oxidase, a copper dependent enzyme (4), and the effects of their molecular constitution on interaction with the enzyme.

In general, all metal complexing agents or compounds which form relatively nondissociated salts with the copper proteinate were found to inactivate the enzyme to some degree. A sulfur atom in the thiol form or compounds capable of tautomeric conversion to the thiol form seemed to be essential for high inhibitory activity among the sulfur-containing compounds. Inhibitory activity varied, however, depending upon the nature of substituents other than sulfur.

Fungitoxic action of certain of the compounds against *Aspergillus niger* seemed to be correlated in a general way with their action against the enzyme whereas no correlation was observed in cases of *Botrytis cinerea*, *Penicillium italicum*, or *Rhizopus nigricans*.

MATERIALS AND METHODS

Polyphenol oxidase was extracted from the commercial mushroom, *Agaricus campestris* L., according to the method of Tenenbaum and Jensen (11). The second extract was dialyzed, buffered at pH 7.0 and kept under refrigeration until used. Activity, based on oxygen uptake, was determined

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by standard Warburg techniques at 30° C. The final volume of test systems was 4 ml. The main compartment of the flasks contained 2 ml. of test chemical solution or suspension, 1 ml. of 1/15 *M* phosphate buffer, pH 6.8, and 0.5 ml. of enzyme solution diluted to catalyze uptake of 300 to 400 μ l. oxygen per hour. The side arm contained 0.5 ml. of 2 per cent pyrocatechol solution which was mixed with the contents of the main compartment after equilibration of the system. Oxygen consumption was recorded at 5-minute intervals over a 30-minute period. Contents of the vessels were continually agitated by the Warburg shaker operating at 100 strokes per minute.

Test chemical solutions or suspensions were prepared by dissolving the chemicals in a small volume of acetone and then diluting them with water to the desired concentration. All chemicals were preincubated with the enzyme for two hours before addition of pyrocatechol. Inhibition values were corrected for autoxidation of catechol.

RESULTS

EFFECTS OF TIME AND CATECHOL ON INHIBITION OF POLYPHENOL OXIDASE BY FUNGICIDES AND RELATED COMPOUNDS

The reaction course of the enzyme preparation on catechol is shown in Figure 1. In accord with the results of other workers (2, 4, 6), oxygen uptake was found to decline at a rapid rate upon addition of catechol to the

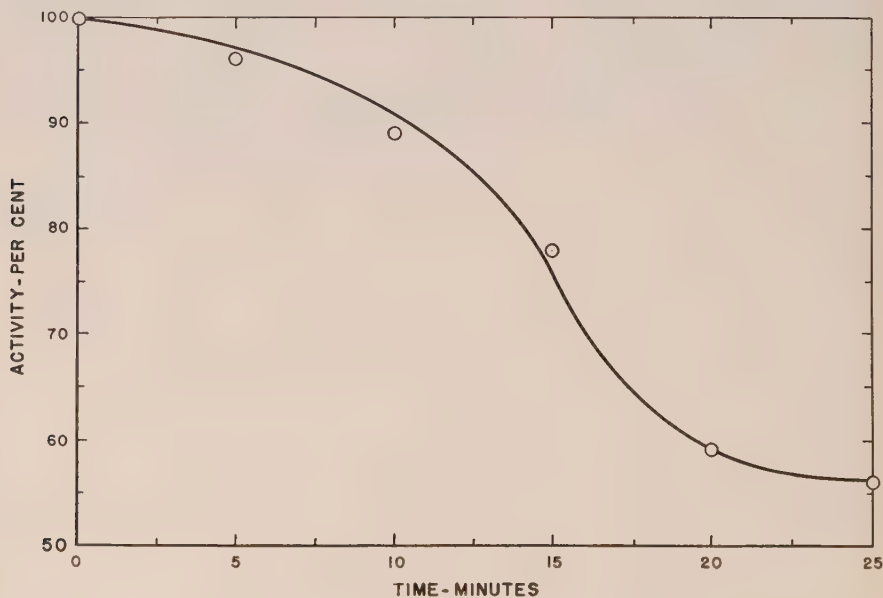


FIGURE 1. Reaction inactivation of polyphenol oxidase.

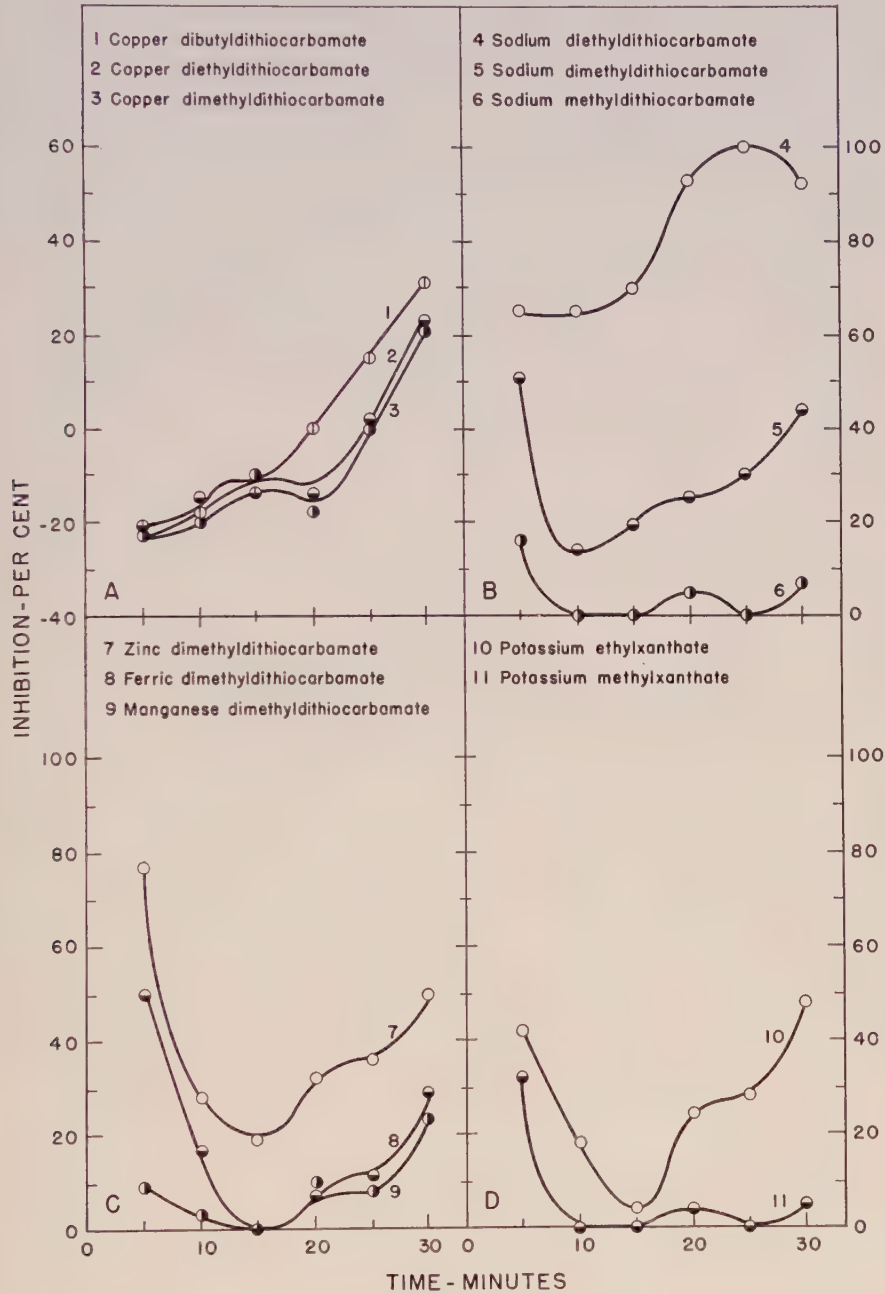


FIGURE 2. Relative time effects of dithiocarbamic acid derivatives at a concentration of 10^{-3} M on polyphenol oxidase activity.

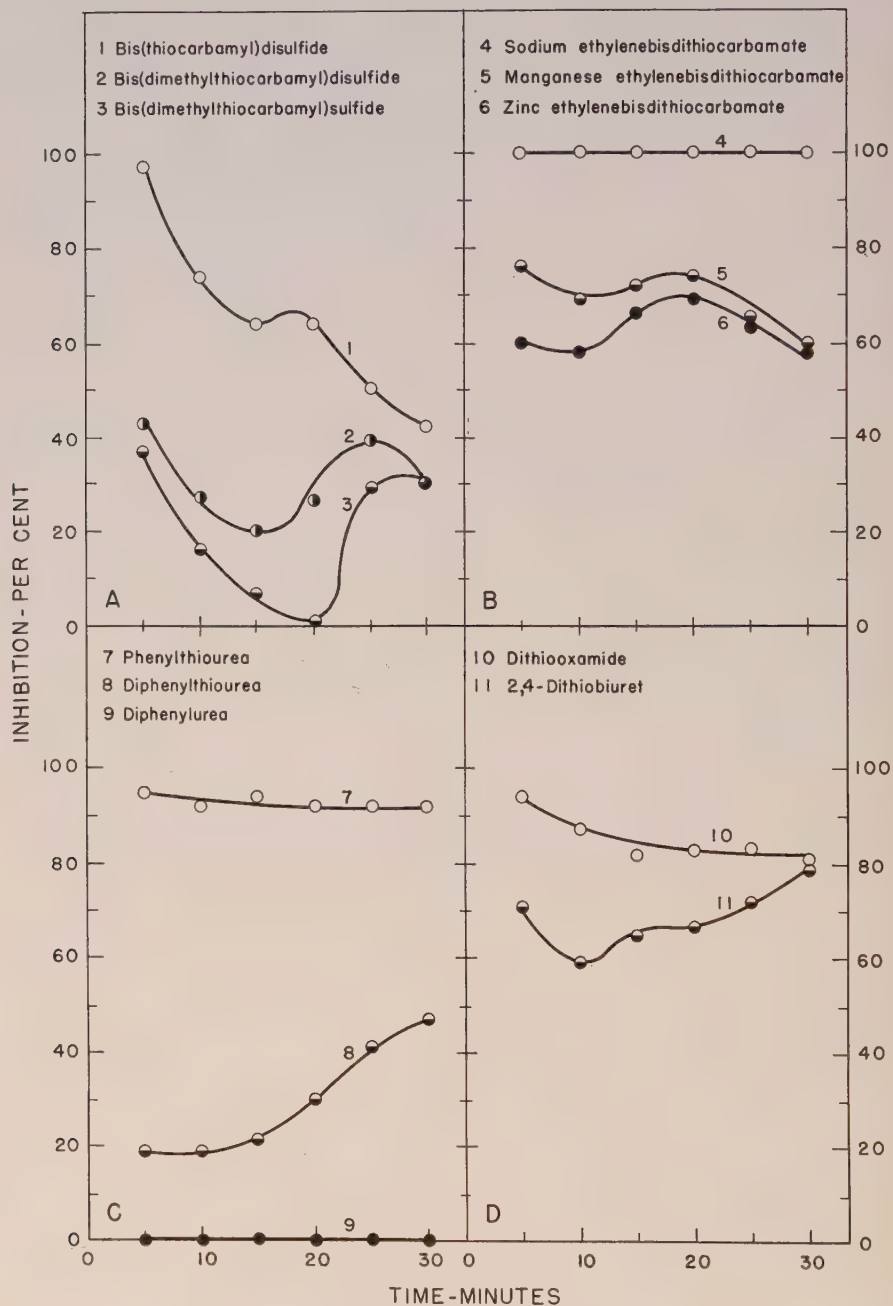


FIGURE 3. Relative time effects of xanthates, bis(thiocarbamyl)sulfides and other metal complexing agents at a concentration of $10^{-3} M$ on polyphenol oxidase activity.

system in the absence of inhibitors. The rate of decline proceeded in a fairly constant manner proportional to time up to about 20 minutes. Thereafter the rate decreased more slowly resulting in a change in the slope of the inactivation curve. This apparently autocatalytic inactivation has been referred to by Dawson and his coworkers as "reaction inactivation" (4) and this term will be used in the present paper. Inhibition by fungicides and other compounds was calculated as percentage reduction in activity based upon the reaction inactivation curve so that the inhibition values represent inactivation over that which occurred spontaneously. The time courses of inactivation by the test chemicals are given in Figures 2, 3, and 4.

Copper dithiocarbamates (Fig. 2 A) consistently increased the oxygen uptake during the first 20 minutes of the reaction period, which seemed to confirm the concept of interaction between the copper moiety of the enzyme and dithiocarbamates upon which the present study was initiated. All other dithiocarbamate salts were inhibitory to some degree, as were bisdithiocarbamates and bis(thiocarbamyl)sulfide and disulfide derivatives.

Inhibition profiles produced by dithiocarbamic acid derivatives other than copper salts seemed to follow three more or less distinctive patterns. Dimethyldithiocarbamates (Fig. 2 B and C) and xanthates (Fig. 2 D) caused marked inhibition of the enzyme during the preincubation period, which indicated formation of a complex between the chemical and the enzyme. Inhibition by all salts of this group decreased rapidly, however, following addition of catechol and sometimes was reversed completely. After about 15 minutes, inhibition began to increase and in most cases approached the initial inhibition value after 30 minutes. During the period of increasing inhibition a plateau was consistently evident, which corresponded in time to the change in rate of reaction inactivation (Fig. 1). Inhibition by sodium diethyldithiocarbamate was anomalous in that it was less affected by catechol than was inhibition by other sodium dithiocarbamates.

Inhibition profiles produced by bis(thiocarbamyl)sulfides and disulfides (Fig. 3 A) were similar to those caused by dithiocarbamate salts during the first 15 minutes. The latter half of the curves was distinctly different, however. The small temporary change in slope which gave rise to the plateau noted in case of dithiocarbamates was much more pronounced in case of methylated bis(thiocarbamyl) derivatives. Inhibition reached a maximum at about 25 minutes and then began to decline. Bis(thiocarbamyl)disulfide produced a different profile in which inhibition decreased rapidly for 15 minutes after addition of catechol, then increased only slightly, and during the final period declined at approximately the initial rate.

Ethylenebisdithiocarbamates (Fig. 3 B) were generally more inhibitory and less affected by catechol than other dithiocarbamic acid derivatives.

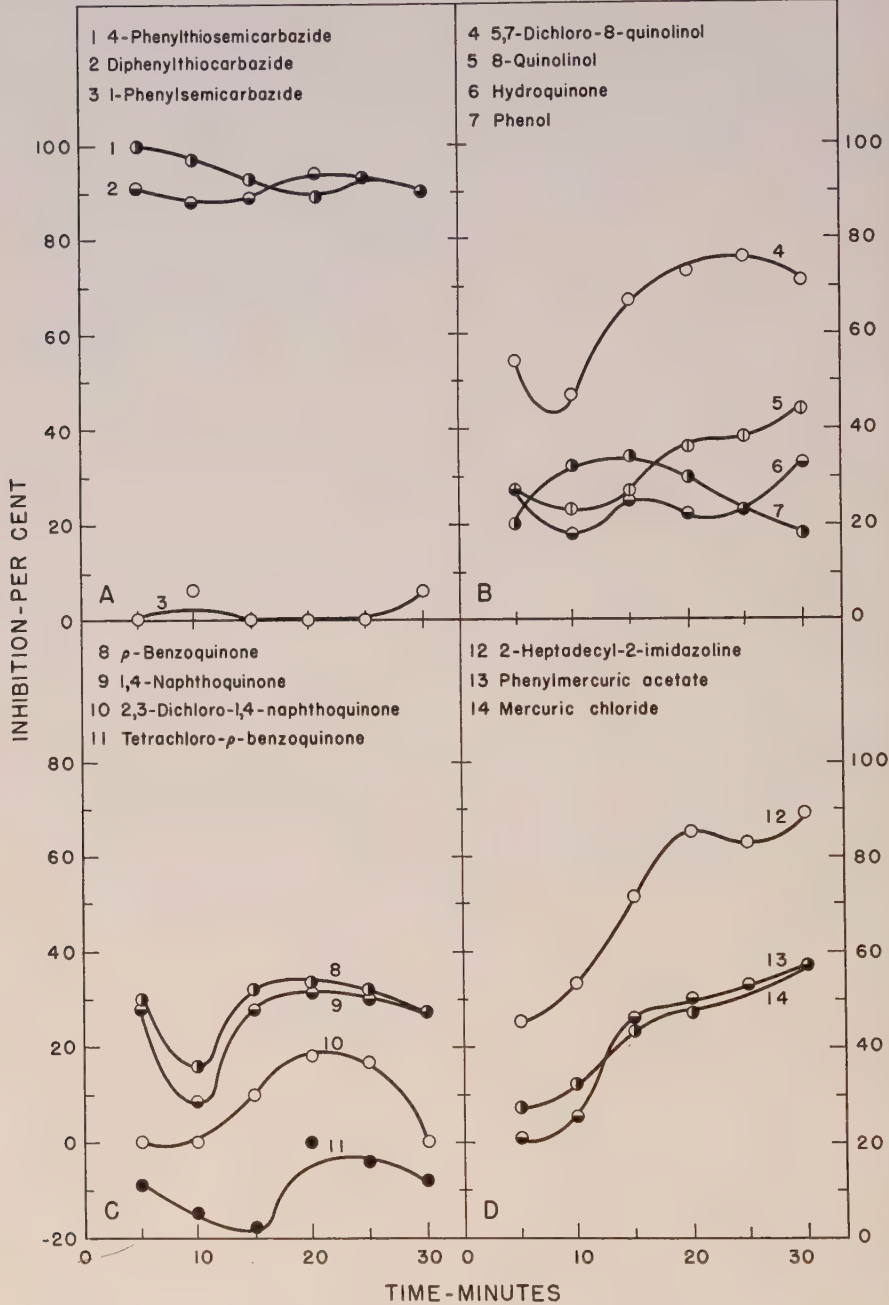


FIGURE 4. Relative time effects of carbazides, phenols, quinones, and protein inactivating compounds at a concentration of 10^{-3} *M* on polyphenol oxidase activity.

The sodium salt was completely inhibitory throughout the test period. With the zinc and manganese salts, however, there was a distinct increase in inhibition at the 20-minute point corresponding to the plateau produced by dithiocarbamate salts, after which inhibition gradually decreased.

Other classes of metal complexing agents produced inhibition profiles with few of the characteristics of dithiocarbamates, except possibly the 20-minute inflection point. Thiourea derivatives (Fig. 3 C) formed stable inhibition complexes with the enzyme. Phenylthiourea was strongly inhibitory and the inhibition complex was apparently unaffected by catechol. Diphenylthiourea was less inhibitory initially but inhibition increased consistently as oxidation proceeded, following in general the reaction inactivation profile. Dithiooxamide and 2,4-dithiobiuret (Fig. 3 D) were very potent inhibitors but inhibition decreased in the early period of the reaction following addition of catechol. Thiocarbazine and thiosemicarbazide derivatives (Fig. 4 A) were among the most inhibitory compounds tested and enzyme complexes formed with these showed no signs of instability in the presence of catechol.

8-Quinololinol and 5,7-dichloro-8-quinolinol (Fig. 4 B) were moderately effective inhibitors. Although there was a slight decrease in initial inhibition following addition of catechol, inactivation increased progressively as the oxidation proceeded.

Inhibition by phenol and hydroquinone (Fig. 4 B) was slight. In case of phenol, which is oxidized by the enzyme but at a slower rate than catechol, inhibition increased with time immediately following addition of catechol, but then began to decrease after 15 minutes. Inhibition by hydroquinone, which is not oxidized by the enzyme, was decreased slightly by addition of catechol, but increased slightly thereafter and produced a distinct inflection in the inhibition profile between 15 and 20 minutes.

Inhibition by unsubstituted benzoquinone and 1,4-naphthoquinone (Fig. 4 C) was of a low order and followed a time course unlike that for sulfur-containing metal complexing agents. Inhibition was decreased sharply immediately following addition of catechol, but rapidly returned to the original value and remained essentially constant for the remainder of the reaction period. Chlorinated quinones, tetrachloro-*p*-benzoquinone, and 2,3-dichloro-1,4-naphthoquinone were noninhibitory initially. Tetrachloro-*p*-benzoquinone increased oxygen uptake initially and up to 20 minutes at which time oxidation was about equal to that of controls. During the remainder of the period there was a slight increase in oxygen uptake. 2,3-Dichloro-1,4-naphthoquinone was somewhat inhibitory after 10 minutes but by the end of the 30-minute period the oxidation rate had returned to a value equal to that of controls.

For comparison with metal complexing agents, several compounds which react with the protein moieties of enzymes were tested (Fig. 4 D).

Inhibition by mercuric chloride and phenylmercuric acetate was similar and increased with time. 2-Heptadecyl-2-imidazoline, a surface active compound, was more inhibitory than either mercury compound but inhibition increased in essentially the same manner, with a distinct inflection in the profile at 20 to 25 minutes. None of the protein reactants were as inhibitory initially after two hours' preincubation with the enzyme as the better metal complexing agents. This suggests that enzyme sites vulnerable to attack by protein precipitants are somewhat inaccessible in case of polyphenol oxidase but that the copper atom is readily accessible to copper complexing agents.

EFFECTS OF CHEMICAL CONSTITUTION ON INHIBITION

Time studies on the course of inhibition in these and previous tests (7) have shown that no single inhibition value can depict accurately the action of an inhibitor since the value changes with time. Values for comparable time periods can be obtained, however, which reflect relative affinities of compounds for the enzyme. In case of polyphenol oxidase the best inhibition value for comparison of different dithiocarbamates appeared to be the initial inhibition value since catechol effects were at a minimum. Initial inhibition values for the various compounds are given in Table I.

The most drastic effects brought about by changes in metal constituents in dithiocarbamates was through the substitution of copper for other metals. Copper resulted in considerable increase in oxygen uptake whereas salts containing other metals were inhibitory. Changes in the dithiocarbamate radical of copper salts caused no appreciable change in their effects on the enzyme. Changes in the dithiocarbamate radical of sodium salts, on the other hand, had marked influence on inhibition. Monomethyldithiocarbamate was least inhibitory. Addition of another methyl group increased inhibitory activity. Replacement of methyl groups by ethyl groups further enhanced inhibitory activity and resulted in a more stable complex with the enzyme. The order of increases in inhibitory activity was in the same direction as increases in inductance effects of methyl and ethyl groups.

Zinc dimethyldithiocarbamate was more inhibitory than either the sodium or iron salt. This was suggestive of the synergistic effect of zinc on sodium ethylenebisdithiocarbamate reported by Barratt and Horsfall (3). However, zinc ethylenebisdithiocarbamate was less active as an inhibitor of the enzyme than was the sodium salt, possibly because of its relatively slight solubility.

Manganese affected the inhibitory properties both of dithiocarbamate and of bisdithiocarbamate adversely when compared to sodium. The adverse effects were undoubtedly related to decrease in solubility and dissociation similar to that noted in case of the zinc salt.

TABLE I
RELATIONSHIPS BETWEEN CHEMICAL CONSTITUTION AND INHIBITION OF POLYPHENOL OXIDASE

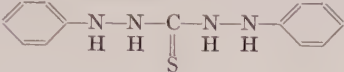
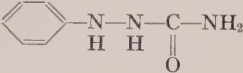

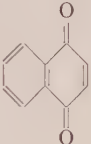
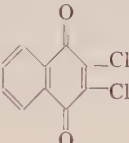
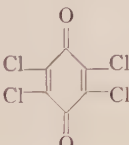
Compound (10^{-3} M)	Formula	Polyphenol oxidase in- hibition (%)
Copper dibutyldithiocarbamate	$\left(\begin{array}{c} \text{C}_4\text{H}_9 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{C}_4\text{H}_9 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_2 \text{Cu}$	19 (+)
Copper diethyldithiocarbamate	$\left(\begin{array}{c} \text{C}_2\text{H}_5 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{C}_2\text{H}_5 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_2 \text{Cu}$	19 (+)
Copper dimethyldithiocarbamate	$\left(\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{CH}_3 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_2 \text{Cu}$	18 (+)
Sodium diethyldithiocarbamate	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \text{Na} \\ \diagup \\ \text{C}_2\text{H}_5 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array}$	65
Sodium dimethyldithiocarbamate	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \text{Na} \\ \diagup \\ \text{CH}_3 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array}$	53
Sodium methyldithiocarbamate	$\begin{array}{c} \text{CH}_3 - \text{N} - \text{C} - \text{S} - \text{Na} \\ \parallel \\ \text{H} \quad \text{S} \end{array}$	16
Zinc dimethyldithiocarbamate	$\left(\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{CH}_3 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_2 \text{Zn}$	77
Ferric dimethyldithiocarbamate	$\left(\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{CH}_3 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_3 \text{Fe}$	50
Manganese dimethyldithiocarbamate	$\left(\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{N} - \text{C} - \text{S} - \\ \diagup \\ \text{CH}_3 \end{array} \begin{array}{c} \parallel \\ \text{S} \end{array} \right)_2 \text{Mn}$	9
Sodium ethylenebisdithiocarbamate	$\text{Na} - \text{S} - \begin{array}{c} \parallel \\ \text{S} \end{array} \text{C} - \text{N} - \text{CH}_2 - \text{CH}_2 - \text{N} - \begin{array}{c} \parallel \\ \text{S} \end{array} \text{C} - \text{S} - \text{Na}$ $\quad \quad \quad \text{H} \quad \quad \quad \text{H}$	100

(+) Indicates increase in oxygen uptake over controls.

TABLE I (Continued)

Compound (10^{-3} M)	Formula	Polyphenol oxidase in- hibition (%)
Manganese ethylenedisithiocarbamate	$\left(\begin{array}{c} \text{---S---C---N---CH}_2\text{---CH}_2\text{---N---C---S---} \\ \parallel \quad \quad \quad \parallel \quad \quad \quad \parallel \\ \text{S} \quad \quad \quad \text{H} \quad \quad \quad \text{H} \quad \quad \quad \text{S} \end{array} \right)_{\text{Mn}}$	76
Zinc ethylenedisithiocarbamate	$\left(\begin{array}{c} \text{---S---C---N---CH}_2\text{---CH}_2\text{---N---C---S---} \\ \parallel \quad \quad \quad \parallel \quad \quad \quad \parallel \\ \text{S} \quad \quad \quad \text{H} \quad \quad \quad \text{H} \quad \quad \quad \text{S} \end{array} \right)_{\text{Zn}}$	60
Potassium ethylxanthate	$\text{C}_2\text{H}_5\text{---O---C---S---K}$ \parallel S	42
Potassium methylxanthate	$\text{CH}_3\text{---O---C---S---K}$ \parallel S	32
Bis(thiocarbamyl)disulfide	$\text{H}_2\text{N---C---S---S---C---NH}_2$ $\parallel \quad \quad \quad \parallel$ $\text{S} \quad \quad \quad \text{S}$	97
Bis(dimethylthiocarbamyl)- disulfide	$\begin{array}{c} \text{CH}_3 \quad \quad \quad \text{CH}_3 \\ \diagdown \quad \diagup \quad \quad \diagdown \quad \diagup \\ \text{N---C---S---S---C---N} \\ \parallel \quad \quad \quad \parallel \\ \text{S} \quad \quad \quad \text{S} \\ \diagup \quad \diagdown \quad \quad \diagup \quad \diagdown \\ \text{CH}_3 \quad \quad \quad \text{CH}_3 \end{array}$	43
Bis(dimethylthiocarbamyl)- sulfide	$\begin{array}{c} \text{CH}_3 \quad \quad \quad \text{CH}_3 \\ \diagdown \quad \diagup \quad \quad \diagdown \quad \diagup \\ \text{N---C---S---C---N} \\ \parallel \quad \quad \quad \parallel \\ \text{S} \quad \quad \quad \text{S} \\ \diagup \quad \diagdown \quad \quad \diagup \quad \diagdown \\ \text{CH}_3 \quad \quad \quad \text{CH}_3 \end{array}$	37
Phenylthiourea	$\begin{array}{c} \text{C}_6\text{H}_5\text{---N---C---NH}_2 \\ \quad \quad \parallel \\ \quad \quad \text{S} \end{array}$	95
Diphenylthiourea	$\begin{array}{c} \text{C}_6\text{H}_5\text{---N---C---N---C}_6\text{H}_5 \\ \quad \quad \parallel \quad \quad \parallel \\ \quad \quad \text{S} \end{array}$	19
Diphenylurea	$\begin{array}{c} \text{C}_6\text{H}_5\text{---N---C---N---C}_6\text{H}_5 \\ \quad \quad \parallel \quad \quad \parallel \\ \quad \quad \text{O} \end{array}$	0
Dithiooxamide	$\text{H}_2\text{N---C---C---NH}_2$ $\parallel \quad \quad \parallel$ $\text{S} \quad \quad \text{S}$	94
2,4-Dithiobiuret	$\text{H}_2\text{N---C---N---C---NH}_2$ $\parallel \quad \quad \parallel$ $\text{S} \quad \quad \text{S}$	71
4 Phenylthiosemicarbazide	$\begin{array}{c} \text{NH}_2\text{---N---C---N---C}_6\text{H}_5 \\ \quad \quad \parallel \\ \quad \quad \text{S} \end{array}$	100

TABLE I (Continued)

Compound (10^{-3} M)	Formula	Polyphenol oxidase in- hibition (%)
Diphenylthiocarbazide		91
1-Phenylsemicarbazide		0
<i>p</i> -Benzoquinone		28
1,4-Naphthoquinone		30
2,3-Dichloro-1,4-naphthoquinone		0
Tetrachloro- <i>p</i> -benzoquinone		(9+)

Bis(thiocarbamyl)disulfide was strongly inhibitory during the first five minutes of the reaction period, but the enzyme-bis(thiocarbamyl)disulfide complex was unstable in the presence of catechol, as evidenced by more than 50 per cent decrease in inhibition in 30 minutes. Addition of methyl groups to form bis(dimethylthiocarbamyl)disulfide reduced the initial inhibition, possibly because of reduced solubility, but the final inhibition value was almost equal to the initial value. If the disulfide was changed to a monosulfide the initial or final inhibition values were not affected although there was considerably more fluctuation in the intermediate time values for the latter compound.

Replacement of the methylamine portion of methylthiocarbamate with a methoxy group to form xanthate resulted in increased inhibition. An ethoxy group further enhanced inhibitory activity in a manner analogous to the effects of ethyl substituents in the dithiocarbamate series.

Inhibition and complex stability in case of dithiooxamide approached the values obtained for ethylenebisdithiocarbamate. Separation of the carbon atoms by nitrogen to form dithiobiuret resulted in decreased activity.

Phenylthiourea was also strongly inhibitory. Addition of another phenyl group to form symmetrical diphenylthiourea greatly decreased inhibitory activity. This suggests that the amino group of dithiooxamide, dithiobiuret and phenylthiourea might be important in the activity of all of these compounds, since the degree of inhibition followed in general the relative ease of tautomeric conversion of these compounds to a thiol form which is capable of forming a mixed complex with the copper proteinate.

Replacement of hydrogen in quinone nuclei by chlorine rendered these compounds noninhibitory. This was in contrast to the augmentation of inhibitory properties of quinones by chlorine substitution reported previously for sulfhydryl- and amino-dependent enzymes (7, 8). Additional studies are necessary to determine whether the increased oxygen uptake observed with tetrachloro-*p*-benzoquinone was due to stimulated enzyme activity, oxidation of quinone by the enzyme, or to increased nonenzymatic oxidation.

POSSIBLE RELATIONSHIPS BETWEEN POLYPHENOL OXIDASE INHIBITION AND FUNGITOXICITY

Initial polyphenol oxidase inhibition caused by several dithiocarbamates and xanthates was compared with fungitoxicity data obtained by Klöpping (5) for several species of fungi. The correlation scattergram of enzyme inhibition plotted against the minimum concentration of fungicide required to inhibit spore germination completely is shown in Figure 5.

Effects of the compounds on spores of *Aspergillus niger* appeared to be roughly parallel to their effects on polyphenol oxidase. Their effects on *Rhizopus nigricans*, while highly variable, suggest that possibly some of the compounds may have parallel activity on spores and polyphenol oxidase. The other species appeared to be in no way correlated even though copper has been shown to be beneficial for their growth (10).

DISCUSSION

While the effects of chemical constitution of dithiocarbamic acid derivatives on polyphenol oxidase inhibition are generally clear and consistent, effects on fungitoxicity are considerably more obscure. If the action of 8-quinolinol is taken as a criterion for comparison of activity, data reported here show that certain of the dithiocarbamates, bisdithiocarbamates, and

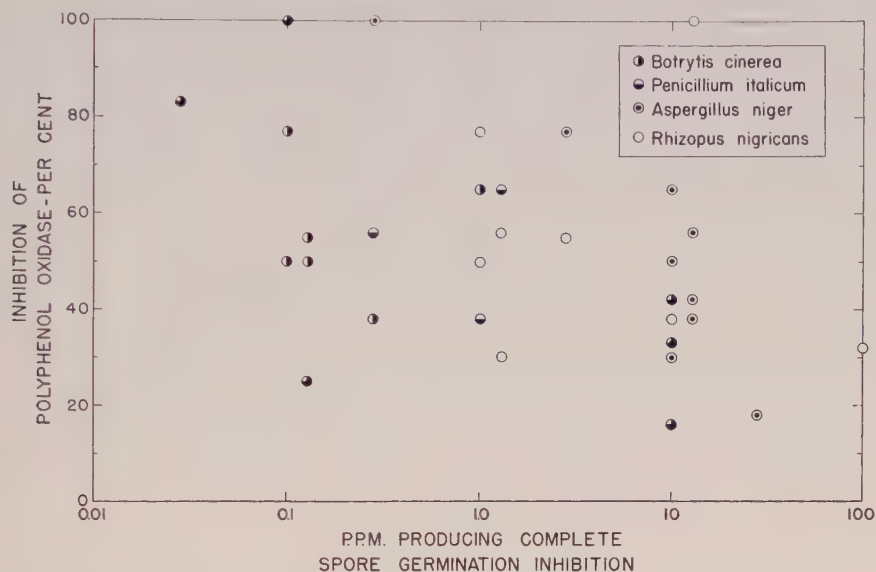


FIGURE 5. Correlation scattergram of relationships between polyphenol oxidase inhibition and fungitoxicity produced by dithiocarbamic acid derivatives.

other compounds are little more effective in inactivating the enzyme than 8-quinolinol. Against certain fungi, such as *Penicillium italicum* and *Botrytis cinerea*, however, zinc and iron dithiocarbamates are as much as 50 times more effective than 8-quinolinol (5). On the other hand, they are about equal to 8-quinolinol against *Aspergillus niger*. This suggests that metal inactivation may be a secondary toxic action of dithiocarbamates against certain fungi, but the principal mode of action against others. It suggests further, as pointed out by Klöpping (5) and Barratt and Horsfall (3), that decomposition intermediates may in some cases play a more important role in toxicity than the original form of the compound. It is becoming increasingly clear, therefore, that no single enzyme relationship exists between most fungicides and spore germination inhibition. Practically all chemicals reported here and in previous papers (7, 8) are decidedly nonspecific and have the capacity to interact with many enzymes, some of them containing fundamentally different catalytic groups. The probability that decomposition intermediates of certain compounds also interact with enzymes adds further to the already tremendous range of possible toxic mechanisms.

Attempts to translate data dealing with isolated enzyme types and fungicides to *in vivo* conditions are also complicated by many factors some of which have been defined previously by many workers. Recent work by Wosilait and Nason (12) and by Rich and Horsfall (9) emphasize some ad-

ditional stumbling blocks that stand in the way of clear cut relationships between *in vitro* and *in vivo* action, i.e., detoxification of chemicals by enzymes or other substances in the cell. The present data indicate further that enzymes inactivated by toxic chemicals sometimes may be restored to activity by the action of cell materials, in some cases the enzyme substrate itself, available from metabolic reservoirs and enzymatic action.

SUMMARY

1. Tests on a series of dithiocarbamates, bisdithiocarbamates, bis(thiocarbamyl)sulfides and disulfides showed that complexes sometimes capable of being dissociated by catechol were formed between the compounds and the enzyme.

2. All dithiocarbamic acid derivatives except copper salts were more or less inhibitory. Several metal complexing agents other than dithiocarbamic acid derivatives were found to be as inhibitory as dithiocarbamates and to form more stable complexes with the enzyme.

3. The principal effects of chemical constitution on dithiocarbamate inhibition of the oxidase were correlated with stability of the molecule and the enzyme-inhibitor complex which were probably due to inductance effects of substituents on the amine nitrogen which affected metal ion dissociation, and to the sulfur atom capable of tautomeric conversion to a sulphydryl form.

4. Based on fungitoxicity data obtained by Klöpping (5), satisfactory correlation between fungitoxicity of dithiocarbamates and their inhibition of polyphenol oxidase was obtained for *Aspergillus niger* whereas no correlation seemed to exist for *Penicillium italicum*, *Botrytis cinerea*, or *Rhizopus nigricans*.

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IMPROVED APPARATUS FOR THE DISTILLATION OF FLUORINE AS HYDROFLUOSILICIC ACID

R. MAVRODINEANU AND J. GWIRTSMAN

In the Willard and Winter method (5) for the volumetric determination of fluorine, its separation is accomplished by steam distillation at a temperature of 135° C. in the presence of perchloric acid. Their method made use of a conventional distilling apparatus consisting of a flask closed with a two-hole rubber stopper through which pass a thermometer and a capillary tube extending into the liquid. The capillary tube is connected to a dropping funnel so that water may be added during the distillation. The distilling flask is connected to a condenser and the solution is heated and distilled. By allowing water to run into the flask from the dropping funnel the temperature is kept constant during the distillation.

Several authors have suggested different procedures to improve this operation. Reynolds, Kershaw, and Jacob (3) described a multiple still for steam distillation of hydrofluosilicic acid; Churchill (1) described a convenient 12-flask distilling unit; Willard, Toribara, and Holland (4) proposed an automatic, electronically operated unit which controlled the flow of the water from the dropping funnel and kept the temperature constant during the distillation; and Huckabay, Welch, and Metler (2) used superheated steam in an apparatus of a different design.

This report describes an improved apparatus for the steam distillation of fluorine as hydrofluosilicic acid from a perchloric acid solution. This equipment has been used continuously for more than one year in this laboratory.

DESCRIPTION OF THE APPARATUS

The distilling unit (Fig. 1 A and B) is composed of a steam generator made from a 1500-ml. flask provided with a safety tube and an outlet which can be closed by a Hoffman clamp. A ground glass joint, J₂ (F 34/45), connects the steam generator with the distilling flask; a second joint, J₁ (F 34/45), connects the latter to a Friedrich type condenser (#3440, Corning Laboratory Glassware Catalog #LP-31).

The distilling flask, D (Fig. 1 B), is made from a Pyrex cylinder (ID, 65 mm.; OD, 70 mm.) through which a thermometer, T, (150° C.) passes by means of an opening (ID, 9 mm.; OD, 11 mm.) and dips into the solution. The steam coming from the steam generator enters through the inlet tube, IT (length, 115 mm.; ID, 6 mm.; OD, 10 mm.), open at both ends, passes through the external tube, OT (length, 120 mm.; ID, 17 mm.; OD,

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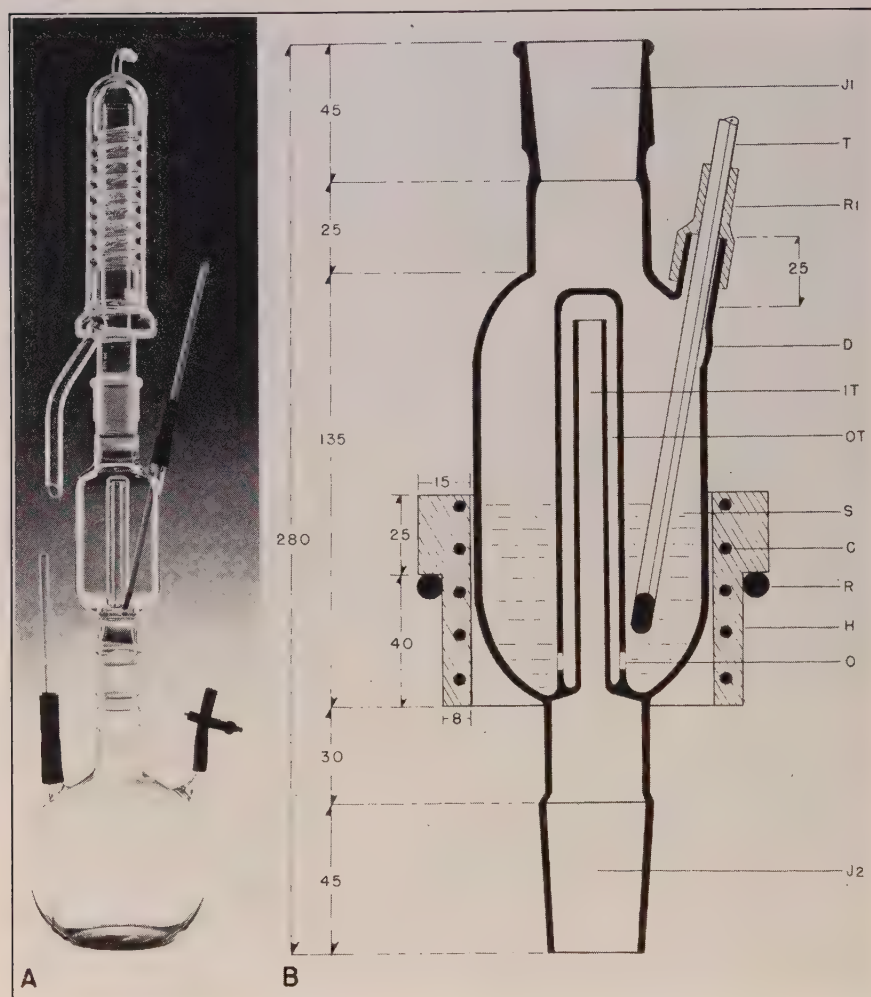


FIGURE 1. (A) Distilling unit. (B) Distilling flask section. The dimensions are given in millimeters. (Explanation in text.)

20 mm.), closed at the top and provided with two openings, O (diameter 5 mm.) at the bottom. After bubbling through the solution, S, the steam condenses in the Friedrich condenser, the distillate being collected in a polyethylene bottle of 250- or 500-ml. capacity.

In order to keep the temperature of the distilling liquid at $135^{\circ} \pm 2^{\circ}$ C. an electric jacket, H, is provided (ID, 72 mm.; OD, upper, 102 mm., lower, 88 mm.). This is made from a nichrome wire spiral, C, having a resistance of 2.120 ohms per ft. and a total length of 6 m. In order to provide ter-

minals, the wire is bent three times and twisted to insure strength over a length of 30 cm. at each end so that the actual resistance length used for the heating is 420 cm. The nichrome spiral is wound on a wooden cylinder (diameter 70 mm.) previously covered with a sheet of asbestos fixed at both ends by two copper wires, and covered with Insalute cement (Fisher Catalog #4-760) in order to obtain the form shown in Figure 1 B. The jacket is then dried for 24 hours in a dry oven at 100° C., taken off the wooden form, and fixed with a metal ring, R, on the frame which supports the entire apparatus (Fisher Flexaframe 14-666-2 plus three additional rods 24 in. long, on which are fixed six rings #14-050, ID, 95 mm.; OD, 102 mm.; and six Castaloy clamps #5-774 with rubber sleeves). The heating jacket is connected to a powerstat (type 116, 0-135 volts and 7.5 amperes supplied by The Superior Electric Co., Bristol, Conn.) which allows the temperature to be regulated. At the beginning of the distillation the powerstat is set to deliver 3.5 amperes and 100 volts, and thereafter the current is decreased to 2 amperes and approximately 60 volts.

The steam generators are heated by individual electric hot plates of 1000 w. (Chromalox hot plates furnished by Edwin L. Wiegand Co., Pittsburgh, Pa.).

If the laboratory is supplied with a steam line, the distilling unit described above may be simplified by omitting the hot plate and the steam generator. In this case the distilling flask will consist simply of a cylindrical container closed at one end, provided with a lateral tube extending almost to the bottom for the introduction of the steam and connected at the other end by means of a ground glass joint, $\text{F } 34/45$, to the Friedrich condenser. The heating jacket will assume the form of a cylinder closed at the bottom and will provide a support for the entire distilling unit.

Figure 2 shows a multiple distilling apparatus consisting of six units. The method of loading the distilling flask with the material to be distilled is shown at the left. In this operation the flask is supported by a copper coil cooled with tap water. The unit on the right is shown in position for washing, which is accomplished in a very convenient way by connecting the base of the distilling flask to a water pump through a ground glass joint, $\text{F } 34/45$, which provides at the same time a rigid support. The outlet tube of the condenser is fitted with a rubber tube dipping into the washing solution (2 l. of boiling fluorine-free water followed by 2 l. of cold fluorine-free water). By starting the water pump the washing liquid is sucked through the entire apparatus, which results in a rapid and thorough cleaning action.

Figure 3 shows a set of six units in operation. The six polyethylene bottles of 500 ml. are supported by a wooden frame fitted with a Flex-board plate in the rear, which provides an efficient thermic insulation and protects the polyethylene bottles from the heat given off by the heating jackets.

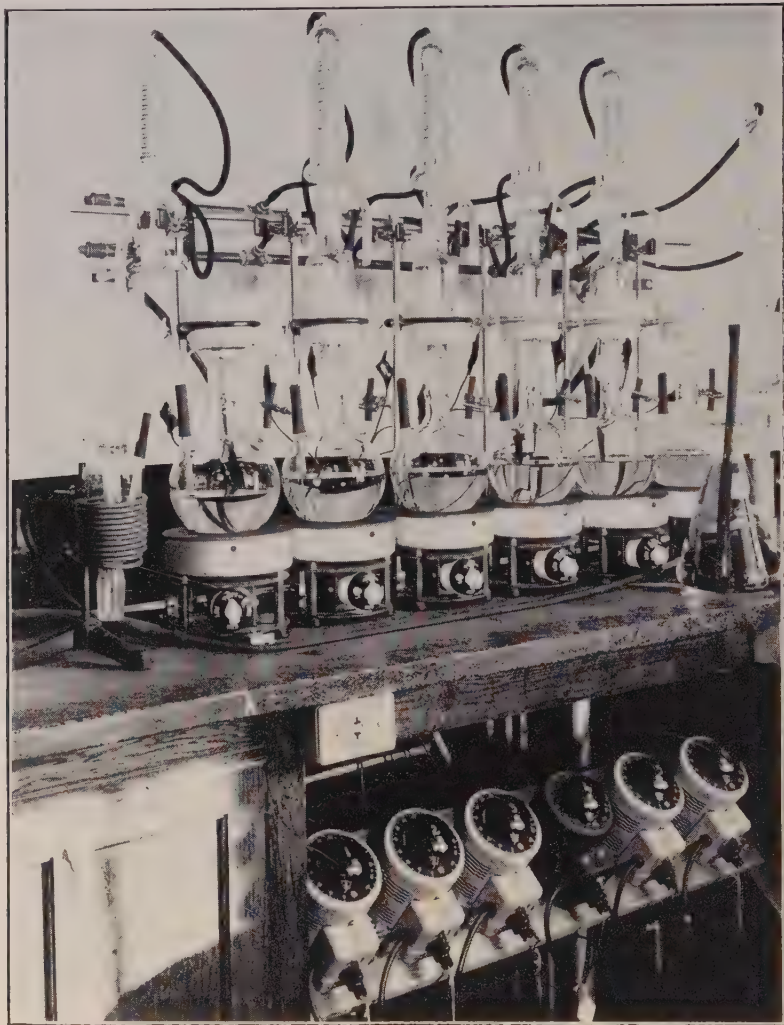


FIGURE 2. Distilling set consisting of six units, shown in various positions.

OPERATION OF THE DISTILLING SET

After the six distilling flasks are loaded and the apparatus is mounted, as shown in Figure 3, the water in the condensers is started, and then the hot plate switches are turned to the position "high" and the powerstat dials set on division 100. When the temperature of the solution in the distilling flask reaches 130°C ., the outlet tube from the steam generators is closed with the Hoffman clamp and the steam allowed to pass through the solution. At the same time the temperature of the heating jackets is re-



FIGURE 3. Distilling set ready for operation.

duced by setting the powerstat dials at division 60. By adjusting the latter, the distilling temperature of $135^{\circ} \pm 2^{\circ}$ C. is easily maintained during the entire operation. The required time for the collection of 500 ml. of distillate is approximately 75 minutes.

After the desired volume of distillate is collected, the Hoffman clamp closing the steam generator is opened, the hot plate and the powerstat turned off, and the cooling water stopped. An entire cycle consisting of loading the distilling flasks, distilling 500 ml., and washing the whole apparatus requires an average time of three hours.

The simplicity in design and operation of this still, as well as the reduced surfaces and connections of the distilling flask and condenser, decreases the possibilities of contamination and losses. The individual steam generators produce a fast distillation, and the complete electric heating system provides a convenient and safe operation. By this means the temperature of $135^{\circ} \pm 2^{\circ}$ C. can be easily maintained in the distilling flask, the heating jacket providing at the same time a good thermal insulation so that the still can be operated with open windows or with a fan blowing close to it. At the end of the distillation the distilling flask and the condenser can be washed together as described above, the procedure being simple, efficient, and automatic.

In order to check the functioning of the new distilling apparatus, a large number of distilling blanks and recovery samples were analyzed. An average blank of 2 μ g. of fluorine and a high recovery averaging 98 to 101 per cent were found. Two distilling apparatuses, each consisting of six units, have been installed in this laboratory and have functioned continuously for more than a year giving complete satisfaction.

SUMMARY

A new type of steam distilling apparatus for the distillation of fluorine as hydrofluosilicic acid is described. Its construction insures a short distillation time of about 75 minutes for the collection of 500 ml. of distillate, low distilling blanks (2 μ g. F), good recovery (98 to 101 per cent), and automatic, efficient washing. The electric heating system provides a convenient and safe operation and maintains a temperature of $135^{\circ} \pm 2^{\circ}$ C. in the distilling flask.

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IMPROVEMENT OF PLANTAGO FOR MUCILAGE PRODUCTION AND GROWTH IN THE UNITED STATES

CLYDE CHANDLER¹

According to the Foreign Trade Division of the United States Department of Commerce 3,808,863 pounds of psyllium (*Plantago*) seed valued at \$1,298,488 were imported into the United States in 1953. Of this total 3,773,102 pounds came from India, 33,600 pounds from Japan, and about 2,161 pounds from France.

Not only are the "husks," or mucilaginous seed coatings, of several species of *Plantago* valuable in pharmaceutical preparations but they have been found effective as basic stabilizers in the ice cream industry (2). They have been used in printing and finishing (7) and in setting lotions (8).

Preliminary field tests indicate that plants of *Plantago ovata*, the species grown in India, can be grown in Arizona. Two other species, *P. rhodosperma* and *P. Wrightiana*, which produce seeds with abundant mucilage, are natives of Texas. Of these three species, *P. ovata* is most desirable for commercial production of mucilage since the seeds are light in color (blond psyllium) and the mucilage is easily removed from the seed in the dry form. However, the plants are only 9 to 16 inches tall and have weak stems which makes it necessary to harvest the seeds by hand. Until these plants are improved through selection and hybridization for types which can be harvested with machinery, commercial production in the United States of America cannot compete with that of India where hand labor is cheap.

Seeds of *Plantago Wrightiana* are also light in color but the mucilage is not so easily removed from the seed as in the case of *P. ovata* except by wet extraction. *P. Wrightiana* plants are approximately 12 to 18 inches tall and more robust than *P. ovata*.

Seeds of *P. rhodosperma* have abundant mucilage but due to the red color in the seed coat a special method for the removal of the mucilage and a bleaching process would have to be developed before it would be acceptable commercially. The large number of seeds per spike makes this species worthy of consideration as material for genetical studies.

In 1951 a project was initiated in this laboratory for the improvement of *Plantago* through selection, hybridization, and induction of ploidy. Tetraploidy was induced in three species. All polyploid plants were more robust than the diploids. Seed size and quantity of mucilage were greater in polyploids but somewhat reduced fertility was found in tetraploid *P. ovata*.

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MATERIAL AND METHODS

Seeds of *Plantago ovata* Cav., *P. rhodosperma* Decne. and *P. Wrightiana* Decne. were used. The last two species (1948 University of Texas crop) and *P. ovata* (1942 Indian crop) had been in dry storage for several years but were still viable as previously reported (1). Untreated seeds were planted in soil and all plants were grown in the greenhouse. Seeds were soaked in aqueous solutions of colchicine for the induction of polyploidy. Concentrations of 0.1 to 2.0 per cent and soaking times of 1 to 24 hours in the laboratory were used.

Flower behavior was observed for plants of the three species. Inter-specific hybridization pollinations were made by carefully emasculating all flowers on plants used as seed parents before pollen was shed and covering emasculated flowers with small frankfurter casings cut in desired lengths. The top of the casing was tied with string while nonabsorbent cotton was inserted in the bottom opening around the peduncle. Flowers on an entire spike were pollinated with pollen of one plant selected as the pollen parent in order to avoid injury from tagging individual flowers. Pollinations were made when the papillae on the stigma were expanded and moist. Bags were removed for pollination but were replaced as soon as pollinations were completed. A Tele-Specta Loupe, commonly used by ophthalmologists, was very helpful in emasculating the small flowers.

Pollen size was used to locate possible polyploids in a population. Positive identification of diploid and tetraploid plants of the three species was made later by the determination of chromosome numbers. The acetic-orcein smear technique (4) was used. Chromosome counts were made at the second anaphase stage of meiosis and from a polar view of the equatorial plate prophase in root tips.

Reduction in fertility accompanying tetraploidy was determined by a study of the number of flowers and the seed produced per spike.

The quantity of mucilage for diploids and tetraploids of the three species was measured by the method given in the 24th edition of The Dispensatory of the United States of America (6, p. 901). One gram of seed was weighed from both diploid and tetraploid lots of each species and placed in 20 ml. of water in a 25-ml. graduated cylinder. Seeds were agitated at intervals during the first 24 hours and then they were left undisturbed for the following 12 hours after which time a reading was taken of the space occupied by the seeds and their mucilage. The number of seeds per gram and the percentage increase in weight of tetraploids were determined.

RESULTS AND DISCUSSION

Flower behavior. The present study shows that flowers of *P. ovata* may be divided into two classes. Of the 1334 plants grown in the greenhouse 866 (65 per cent) were almost completely male sterile (Fig. 1 A and D). All an-



FIGURE 1. Flower types: (A) Flowers of male sterile diploid *P. ovata* showing (left to right) protruding style before expansion of corolla, small anthers of fully opened flower, and elongated style typical of older flowers ($\times 3.1$). (B) Flowers of fertile diploid *P. ovata* showing unopened flower with style protruding slightly, large anthers of fully expanded flower, and short style of older flowers ($\times 3.1$). (C) Flowers of the tetraploid *P. ovata*. Pistil protrudes slightly before flower opens, fully expanded flower with larger anthers, and pistil elongated ($\times 3.1$). (D) Three flowering spikes of *P. ovata* (diploid) showing male sterile flowers at time of anthesis, old flowers with elongated styles also from male sterile plants, and fertile diploid with large anthers with abundant pollen ($\times 0.64$). (E) Three flower spikes of *P. Wrightiana*. Left, pollination occurred in bud; center, large anthers with abundant pollen visible when corolla expands; right, elongated pistils after anthesis ($\times 0.64$).

thers were very small, shriveled and only occasionally were a few pollen grains formed. The other 468 (35 per cent) produced flowers with fully expanded corolla. Large anthers produced on long filaments were filled with abundant pollen of uniform size as shown in Figure 1 B. These plants were highly self-fertile.

The majority of the flowers of *P. Wrightiana* have short stamens approximately the length of the pistil. Pollen sheds and fertilization is accomplished before the corolla expands (Fig. 1 E). The second class has flowers with long stamens which extend beyond the corolla tips. Pollen is shed and fertilization occurs after the flower is fully open. Of 477 plants, 430 belong to the first class and only 47 to the second group. Both types may occur on the same plant and even in the same spike though this is not common for this species.

Flowers of *P. rhodosperma* are for the most part cleistogamic. However, chasmogamic flowers were observed on some plants of this species.

These observations on flower behavior were made to determine when pollinations should be made to obtain viable seed. Further studies would probably permit a more extended classification of flower types. Stout (9) describes three distinct types of flowers for *Plantago lanceolata* and observed many variations of these forms.

Polyploidy. Natural polyploidy has not been reported for any of the three species included in the present study though it has been known to occur in other species (3, 5).

The induction of polyploidy was initiated as a means to increase seed size and vigor of the plant. It was hoped that more mucilage could be obtained from larger seeds. Tetraploidy was induced by soaking seeds in aqueous solutions of colchicine. A wide range in concentration of the solution and the time of treatment was effective. Seeds soaked in concentrations of 2.0 per cent for 2, 8, 12, 20, and 24 hours, 1.0 per cent for 1, 12, 20, and 24 hours, 0.5 per cent for 4, 8, 16, and 24 hours, 0.2 per cent for 8, 16, and 24 hours, and 0.1 per cent for 16, 20, and 24 hours produced some poly-ploids. However, the greater number of poly-ploids was obtained from treatment in 1.0 per cent colchicine for 16 or 20 hours.

Pollen studies were made to locate possible poly-ploids. The size of the pollen grains from tetraploid plants was larger than those from diploids (Fig. 2) and served as a useful tool for locating possible poly-ploids. Flowers were also larger (Fig. 1 C).

The diploid number of chromosomes in *P. ovata* was checked and found to be $2n=8$. The diploid number for *P. Wrightiana* and *P. rhodosperma*, not previously reported, was determined from studies of pollen mother cells and root tips and found to be $2n=20$ and 24 respectively. Acetic-orcein smears of pollen mother cells from plants having larger pollen grains

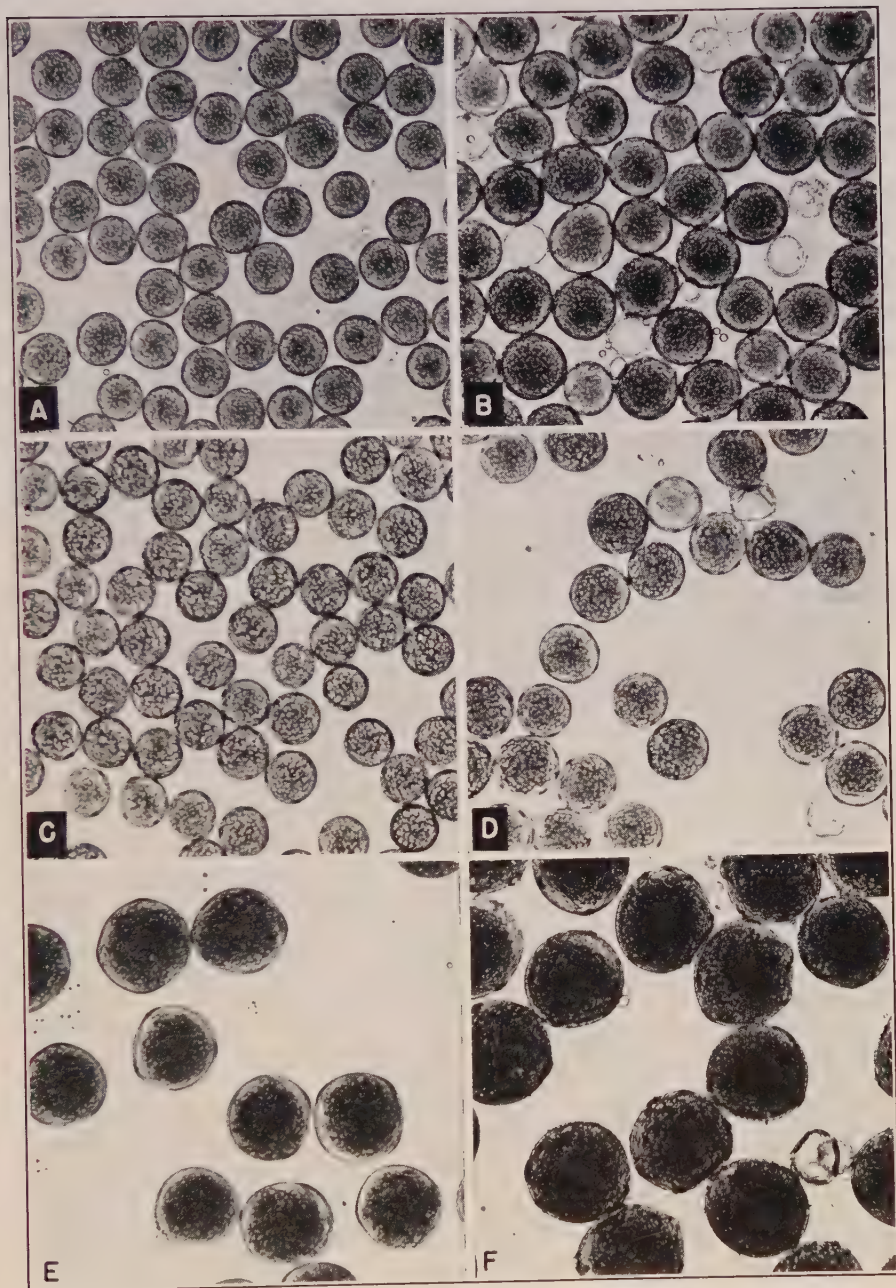


FIGURE 2. Pollen grains. (A, B) *P. ovata*. (C, D) *P. rhodosperma*. (E, F) *P. Wrightiana*. Left, diploids. Right, tetraploids ($\times 225$).

revealed $n = 8$ (one plant *P. ovata*), $n = 20$ (14 plants of *P. Wrightiana*) and $n = 24$ (13 plants of *P. rhodosperma*).

Effect of polyploidy on seed size and mucilage. Tetraploid plants produced larger seed than their corresponding diploids as may be seen in Figures 3 and 4. One gram of seed from the diploid and tetraploid plants of the three species was weighed and then the total number of seeds in each lot was determined. A duplicate sample was weighed for each seed type. The data in Table I show 36.0 per cent increase in weight of tetraploid *P. ovata* seed over the diploid. Tetraploid seeds of *P. Wrightiana* and *P. rhodosperma* were 27.3 per cent larger than the diploid seeds of the same species.

TABLE I
QUANTITY OF MUCILAGE ON DIPLOID VERSUS TETRAPLOID SEEDS OF *PLANTAGO*

Species	Number of seed in 1 gram		Per cent increase in seed weight in tetraploids	Quantity* of mucilage on seed		Per cent increase in mucilage for 1 gram of tetraploid seed
	Diploid	Tetraploid		Diploid	Tetraploid	
<i>P. ovata</i>	525	335	36.2	12.0	15.0	25.0
	530	340	35.8	12.5	15.0	20.0
<i>P. Wrightiana</i>	384	273	28.9	18.5	19.0	2.7
	378	281	25.7	18.5	19.0	2.7
<i>P. rhodosperma</i>	707	505	28.6	14.0	17.5	25.0
	707	520	25.9	14.0	18.0	28.6

* Total volume (ml.) occupied by the swollen seed determined by the standard test given in The Dispensatory of the United States (6, p. 901).

In addition to an increase in seed size of the tetraploids, there was a definite increase in the amount of mucilage surrounding the seed. Tests in duplicate showed 22.5 per cent, 2.7 per cent, and 26.8 per cent increase in mucilage of tetraploids over diploids of *P. ovata*, *P. Wrightiana*, and *P. rhodosperma*.

The increase in seed size and the amount of mucilage as well as vegetative vigor in tetraploids compared with diploids is of decided commercial interest and more than compensates for the reduction in fertility to be described below.

Reduced fertility of tetraploids. Earnshaw (3) reported higher seed production for natural tetraploids than diploids of similar geographic source.

Based upon greenhouse studies only, the induced tetraploids described in this paper showed a reduction in fertility of 26.0, 9.2, and 10.0 per cent for *P. ovata*, *P. Wrightiana*, and *P. rhodosperma* as shown in Table II. In each case flowers and seed were counted from the largest spikes produced. The higher percentage of reduction in fertility of the *P. ovata* tetraploid

TABLE II
FERTILITY OF DIPLOIDS AND TETRAPLOIDS OF THREE SPECIES OF PLANTAGO

Fertility	<i>P. ovata</i>		<i>P. Wrightiana</i>		<i>P. rhodosperma</i>	
	Diploid	Tetra- ploid	Diploid	Tetra- ploid	Diploid	Tetra- ploid
Total No. of spikes	50	50	10	10	25	25
Total No. of flowers	2137	1792	504	564	2081	3805
Total No. of seed	3787	2242	972	984	5040	5667
Av. No. flowers per spike	42.7	35.8	50.4	56.4	119.2	152.2
Av. No. seed per spike	75.6	44.8	97.2	98.4	201.6	226.7
Per cent seed production	88.6	62.6	96.4	87.2	84.5	74.5
Per cent reduced fertility	26.0		9.2		10.0	

may be due in part to a peculiar condition of the tetraploid plant. When young tetraploid seedlings were growing vigorously a dark exudate appeared on the leaves in the form of droplets which spread along the leaf surface. Leaves became unusually brittle and curled. Before seeds matured much of the foliage died. During the summer when days were long, plants were relatively free from this dark exudate. It may result from a physiological condition in tetraploids when growing conditions are less favorable. Further work is contemplated on this phase of the problem. The total number of spikes and seed per plant for *P. ovata* is being investigated for further evaluation of fertility of diploids *vs.* tetraploids.

Seed production. All types of flowers of the three diploid species of *Plantago* set seed abundantly to open pollinations. One hundred and forty-four male sterile plants of *P. ovata* were isolated in a greenhouse where no other *Plantago* were growing. Of the 887 flowers from 44 spikes (average number of flowers per spike, 20.2) only one seed was obtained. Another 2021 spikes were collected and from these spikes 274 seed were produced. If all flowers were fertilized we would expect approximately 80,840 seed. For commercial breeding of *P. ovata* it may be possible to use these semi-sterile plants as seed parents without emasculating all flowers before cross-pollinations are attempted since seed production is only 0.3 per cent of what might normally be expected. Even this slight degree of seed production might be eliminated by covering the entire plant with a cage.

All tetraploid plants of the three species under discussion were also highly fertile to open pollination. The degree of fertility was slightly less than for diploids, as has been indicated.

Breeding. All flowers on 33 spikes of male sterile *P. ovata* crossed with seven different individuals of *P. rhodosperma* failed to yield any seed. Interspecific hybridization pollinations involving all possible combinations of *P. ovata*, *P. rhodosperma*, and *P. Wrightiana* have failed when pollinations were fully controlled. The difference in chromosome numbers, *P.*



FIGURE 3. *Plantago ovata* grown in pots under greenhouse conditions. (A) Left, typical bushy diploid plant compared to tetraploid (on right) with heavier stem, more upright growth, and heavier spikes ($\times 0.13$). (B) Mature seed, spikes, and foliage of diploid (left) and tetraploid (right) ($\times 0.72$).

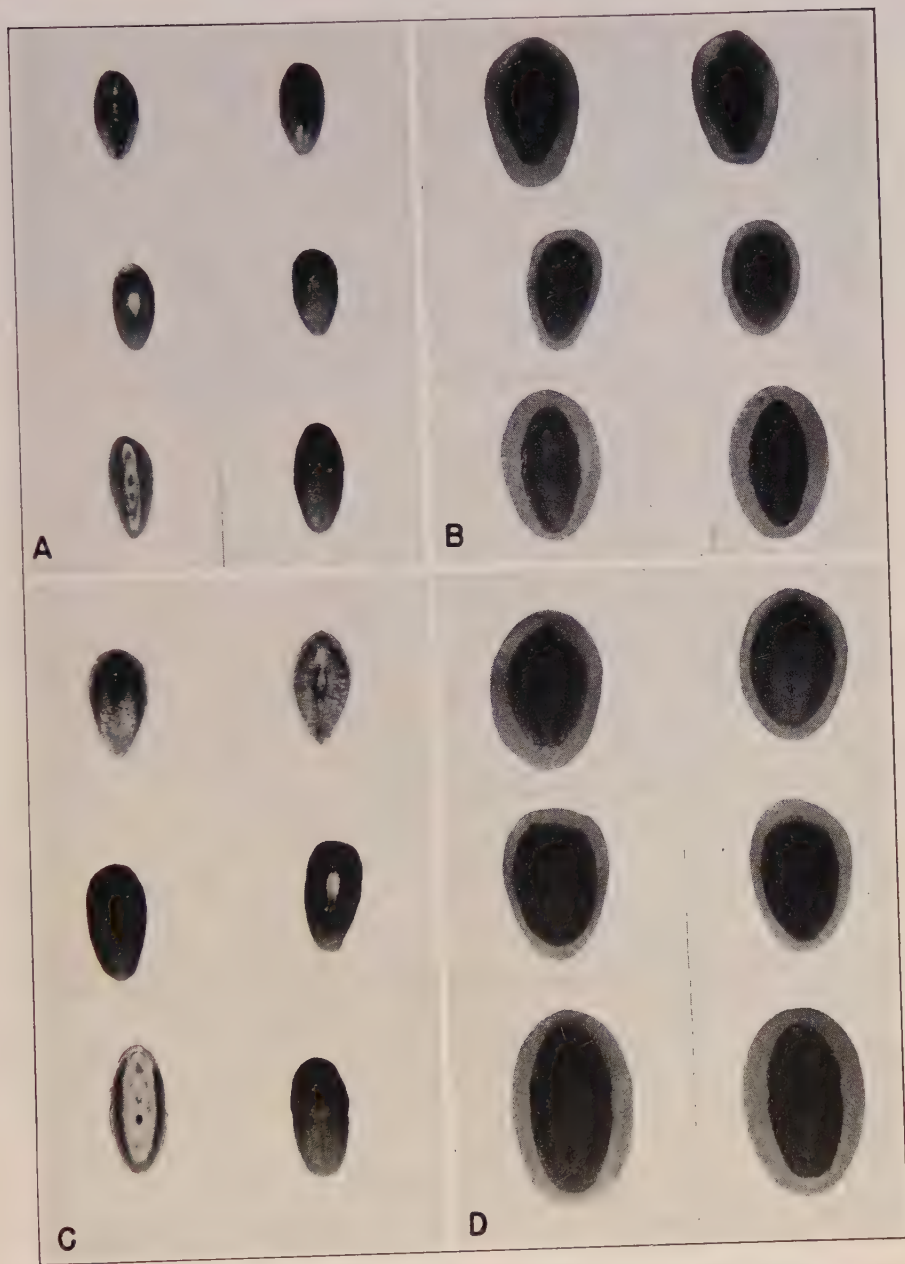


FIGURE 4. Dry and soaked seeds of *Plantago*. (A) Dry diploid seed. Row 1, *P. ovata*; row 2, *P. rhodosperma*; row 3, *P. Wrightiana*. (B) Seeds of same after 16 hrs. on moist filter paper. (C) Dry tetraploid seed. (D) Moist tetraploid seed of above three species ($\times 4$).

ovata $n=4$, *P. Wrightiana* $n=10$, and *P. rhodosperma* $n=12$, may account for these failures in seed production.

Male sterile plants of diploid *P. ovata* crossed with tetraploid *P. ovata* failed completely in seed production while 63 seeds were obtained from fully controlled pollinations of the tetraploid \times fertile diploid. Though this combination was feebly fertile a few seeds were produced. One half of these seed was planted in soil in the greenhouse but none germinated. The other half was planted on filter paper in Petri dishes in the laboratory. None germinated but neither did they mold which indicated that embryos were viable. Embryos were dissected and grown on filter paper but all died when transferred to soil. No doubt these embryos will have to be grown on an artificial medium with proper nutrients supplied until they are sufficiently strong to be transplanted in soil.

Commercial trials. Ten lots of tetraploid *P. ovata*, 95 lots of tetraploid *P. Wrightiana*, and 111 lots of tetraploid *P. rhodosperma* seed were sent out for commercial plantings in Arizona. A good supply of these seed is still available for further tests. The real value of tetraploid plants over diploids cannot be determined until adequate commercial tests are made. However, experimental data indicate that the greater seed size and increase in mucilage around the seeds will make tetraploid plants desirable for the commercial production of psyllium.

SUMMARY

1. Tetraploidy was induced in three diploid species of *Plantago* (*P. ovata* $n=4$, *P. Wrightiana* $n=10$, *P. rhodosperma* $n=12$) by soaking seeds in aqueous solutions of colchicine. One autotetraploid of *P. ovata* $n=8$, 14 autotetraploids of *P. Wrightiana* $n=20$, and 13 of *P. rhodosperma* $n=24$ were obtained. No natural polyploids appeared in any of the progenies grown from untreated seed.

2. Various types of flower behavior were observed for these three species of *Plantago*. Male sterile diploid plants of *P. ovata* were of frequent occurrence.

3. All diploid plants of the three species set abundant seed to open pollination. Fertility of tetraploids was slightly less than in diploids.

4. Seed size, quantity of mucilage, and vigor of plant were noticeably greater in tetraploids.

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